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(21) International Application Number: PCT/US92/01152 (22) International Filing Date: 10 February 1992 (10.02.92) (30) Priority data: 654,205 8 February 1991 (08.02.91) US (60) Parent Application or Grant (63) Related by Continuation US 654,205 (CIP) Filed on 8 February 1991 (08.02.91) (71) Applicant (for all designated States except US): PROGEN- ICS PHARMACEUTICALS, INC. [US/US]; Old Saw Mill River Road, Tarrytown, NY 10591 (US).		(72) Inventors; and (75) Inventors/Applicants (for US only) : BEAUDRY, Gary, A. [US/US]; 109 Inwood Avenue, Upper Montclair, NJ 07043 (US). MADDON, Paul, J. [US/US]; 60 Haven Avenue, Apt. 25c, New York, NY 10032 (US). (74) Agent: WHITE, John, P.; Cooper & Dunham, 30 Rocke- feller Plaza, New York, NY 10112 (US). (81) Designated States: AT (European patent), AU, BE (Euro- pean patent), CA, CH (European patent), DE (Euro- pean patent), DK (European patent), ES (European pa- tent), FR (European patent), GB (European patent), GR (European patent), IT (European patent), JP, LU (Euro- pean patent), MC (European patent), NL (European pa- tent), SE (European patent), US. Published <i>With international search report.</i> <i>Before the expiration of the time limit for amending the</i> <i>claims and to be republished in the event of the receipt of</i> <i>amendments.</i>
(54) Title: CD4-GAMMA1 AND CD4-IgG1 CHIMERAS (57) Abstract This invention provides an expression vector encoding a CD4-gamma1 chimeric heavy chain homodimer. This invention also provides an expression vector encoding the heavy chains of a CD4-IgG1 chimeric heterotetramer. Finally, this invention provides an expression vector encoding the light chains of a CD4-IgG1 chimeric heterotetramer.		

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CD4-GAMMA1 AND CD4-IgG1 CHIMERASBackground of the Invention

Throughout this application, various publications are referenced by Arabic numerals within parentheses. Full citations for these publications may be found at the end of the specification immediately preceding the claims. The disclosures of these publications in their entireties are hereby incorporated by reference into this application in order to more fully describe the state of the art as known to those skilled therein as of the date of the invention described and claimed herein.

The life cycle of animal viruses is characterized by a series of events that are required for the productive infection of the host cell. The initial step in the replicative cycle is the attachment of the virus to the cell surface which is mediated by the specific interaction of the viral attachment protein (VAP) to receptors on the surface of the target cell. The pattern of expression of these receptors is largely responsible for the host range and tropic properties of viruses. The interaction of the VAP with cellular receptors therefore plays a critical role in infection and pathogenesis of viral diseases and represents an important area to target the development of anti-viral therapeutics.

Cellular receptors may be comprised of all the components of membranes, including proteins, carbohydrates, and lipids. Identification of the molecules mediating the attachment of viruses to the target cell surface has been made in a few instances. The most extensively characterized viral receptor protein is CD4 (T4) (1). CD4 is a nonpolymorphic cell surface glycoprotein that is expressed primarily on the surface of helper T lymphocytes and cells of monocyte/macrophage lineage. CD4 associates with ma

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histocompatibility complex (MHC) class II molecules on the surface of antigen-presenting cells to mediate efficient cellular immune response interactions. In man, CD4 is also the target of interaction with the human immunodeficiency virus (HIV).

5 HIV infects primarily helper T lymphocytes and monocytes/macrophages, cells that express surface CD4, leading to a gradual loss of immune function which results in the development of the human acquired immune deficiency syndrome (AIDS). The initial phase of the HIV replicative
10 cycle involves the high affinity interaction between the HIV exterior envelope glycoprotein gp120 and surface CD4 (Kd approximately 4×10^{-9} M) (2). Several lines of evidence demonstrate the requirement of this interaction for viral infectivity. In vitro, the introduction of a functional
15 cDNA encoding CD4 into human cells which do not express CD4 is sufficient to render otherwise resistant cells susceptible to HIV infection (3). In vivo, viral infection appears to be restricted to cells expressing CD4. Following the binding of HIV gp120 to cell surface CD4, viral and
20 target cell membranes fuse, resulting in the introduction of the viral capsid into the target cell cytoplasm.

Characterization of the interaction between HIV gp120 and CD4 has been facilitated by the isolation of cDNA clones
25 encoding both molecules (4, 5). CD4 is a nonpolymorphic, lineage-restricted cell surface glycoprotein that is a member of the immunoglobulin gene superfamily. High-level expression of both full-length CD4 and truncated, soluble versions of CD4 (sCD4) have been described in stable
30 expression systems. The availability of large quantities of purified sCD4 has permitted a detailed understanding of the structure of this complex glycoprotein. Mature CD4 has a relative molecular mass (Mr) of 55 kilodaltons and consists

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of an amino-terminal 372 amino acid extracellular domain containing four tandem immunoglobulin-like regions denoted V1-V4, followed by a 23 amino acid transmembrane domain and a 38 amino acid cytoplasmic segment. The amino-terminal immunoglobulin-like domain V1 bears 32% homology with kappa light chain variable domains. Three of the four immunoglobulin-like domains contain a disulphide bond (V1, V2 and V4), and both N-linked glycosylation sites in the carboxy-terminal portion of the molecule are utilized (4, 6).

Experiments using truncated SCD4 proteins demonstrate that the determinants of high-affinity binding to HIV gp120 lie within the amino-terminal immunoglobulin-like domain V1 (7-9). Mutational analysis of V1 has defined a discrete gp120 binding site (residues 38-52 of the mature CD4 protein) that comprises a region structurally homologous to the second complementarity-determining region (CDR2) of immunoglobulins (9). The production of large quantities of V1V2 has permitted a structural analysis of the two amino-terminal immunoglobulin-like domains. The structure determined at 2.3 angstrom resolution reveals that the molecule has two tightly associated domains containing the immunoglobulin-fold connected by a continuous beta strand. The putative binding sites for monoclonal antibodies, class II MHC molecules and HIV gp120 (as determined by mutational analysis) map on the molecular surface (10, 11).

A soluble version of the entire extracellular segment of CD4 (V1-V4, termed SCD4) has been described and appears to be a potential therapeutic approach to the treatment of HIV infection (12). In vitro experiments demonstrate that: 1) SCD4 acts as a "molecular decoy" by binding to HIV gp120 and inhibiting viral attachment to and subsequent infection of human cells; 2) SCD4 "strips" the viral envelope glycoprotein gp120 from the viral surface; and 3) cCD4

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blocks the intercellular spread of virus from HIV-infected cells to uninfected cells by inhibiting virus-mediated cell fusion (1, 13).

5 In addition to in vitro results, experiments with sCD4 in simian immunodeficiency virus (SIV)-infected rhesus monkeys have been described. These studies demonstrated that administration of 2 milligrams (intramuscular) of sCD4 for 28 days to SIV-infected rhesus monkeys led to a decreased ability to isolate virus from peripheral blood lymphocytes and bone marrow. In addition, the growth of granulocyte-macrophage and erythrocyte progenitor colonies in the bone marrow returned to normal levels. These data suggest that 10 administration of sCD4 to SIV-infected rhesus monkeys leads to a diminution of the viral reservoir.

15 Phase I human clinical trials demonstrated that there is no significant toxicity or immunogenicity associated with administration of sCD4 at doses as high as 30 mg/day. Pharmacokinetic studies revealed the serum half-life of sCD4 to be 45 minutes following intravenous administration, 9.4 20 hours after intramuscular dosing, and 10.3 hours after the drug was given subcutaneously (14, 15). Preliminary antiviral studies were inconclusive with respect to CD4 cell count and levels of HIV antigen. Because the maximum tolerated dose was not reached, the antiviral effect of sCD4 25 may have been underestimated, especially in light of recent data concerning differences in sCD4 concentrations required to inhibit laboratory strains of HIV-1 compared to primary viral isolates (16).

30 Although these in vitro, primate, and human clinical studies with sCD4 have produced encouraging results, they have also defined several limitations. First, the measured serum half-life of sCD4 is relatively short. Second, sCD4 is monovalent with respect to gp120 binding in contrast with 35

cell surface CD4 and viral surface gp120 which are multivalent. Third, sCD4 is not cytotoxic for HIV-infected cells. Fourth, sCD4 may not cross the placenta to a significant degree. Therefore, chimeric CD4 molecules have been described which take advantage of the immunoglobulin-like nature of CD4 and several beneficial properties of immunoglobulins themselves (i.e. CD4-immunoglobulin fusions).

Immunoglobulins, or antibodies, are the antigen-binding molecules produced by B lymphocytes which comprise the humoral immune response. The basic unit of an immunoglobulin molecule consists of two identical heavy chains and two identical light chains. The amino-terminus of each chain contains a region of variable amino acid sequence (variable region). The variable regions of the heavy and light chains interact to form two antigen binding sites. The carboxy-terminus of each chain contains a region of constant amino acid sequence (constant region). The light chain contains a single constant domain, whereas the heavy chain constant domain is subdivided into four separate domains (CH1, hinge, CH2, and CH3). The heavy chains of immunoglobulin molecules are of several types, including mu (M), delta (D), gamma (G), alpha (A) and epsilon (E). The light chains of immunoglobulin molecules are of two types, either kappa or lambda. Within the individual types of heavy and light chains exist subtypes which may differ in effector function. An assembled immunoglobulin molecule derives its name from the type of heavy chain that it possesses.

The development of monoclonal antibodies has circumvented the inherent heterogeneity of antibodies obtained from serum of animals or humans. However, most monoclonal antibodies are derived from cells of mouse origin and therefore are immunogenic when administered to humans. More recent

developments combining the techniques of molecular genetics with monoclonal antibody technology has lead to the production of "humanized" chimeric antibodies in vitro. In these chimeric antibodies, the variable domains of human immunoglobulin heavy and light chains are replaced with specific heavy and light chain variable domains from a murine monoclonal antibody (17-19). The result of this genetic manipulation is a molecule with specificity for a particular antigen and the characteristics of human immunoglobulins.

Sequence and structural analyses of CD4 indicate that the four extracellular domains are immunoglobulin-like. Since the Fc portion of immunoglobulins controls the rate of catabolism of the molecules (serum half-life ranging from 14 to 21 days) and provides various effector functions, several reports describe the replacement of variable and constant domains of immunoglobulins with the immunoglobulin-like domains of CD4 (21-24).

CD4-IgG1 heavy chain fusion proteins resulting in chimeric gamma1 heavy chain dimers have been described (21). These molecules contain the gamma1 heavy chain CH1 domain in addition to the hinge, CH2 and CH3 domains. However, heavy chain assembly and secretion from mammalian cells is less efficient if the CH1 domain is expressed in the absence of light chains (25). Subsequently, a CD4-IgG1 heavy chain fusion protein lacking the CH1 domain and the first five amino acids of the hinge region was described which was secreted to high levels (22). These fusion proteins retain various effector functions of immunoglobulin molecules, such as Fc receptor binding, antibody-dependent cell-mediated cytotoxicity (ADCC) toward HIV-1-infected cells, and placental transfer via an Fc receptor-dependent mechanism (22). CD4-IgM heavy chain fusion proteins have also been described (26). In addition, CD4-IgG1 fusion proteins have

been described wherein the V1V2 domains of CD4 are fused to the CH1, hinge, CH2 and CH3 domains of a gamma1 heavy chain, and wherein the V1V2 domains of CD4 are fused to the constant domain of a kappa light chain (29).

5 Fusion proteins linking CD4 to toxins have also been constructed and tested for their ability to kill HIV-infected cells. In one study, sCD4 was coupled to the deglycosylated A chain of ricin which inactivates ribosomes, therefore inhibiting protein synthesis and killing the cell (27). This fusion protein was reported to specifically lyse
10 cells infected with five different isolates of HIV, but was nontoxic to uninfected cells. In another study, the V1V2 domains of CD4 were coupled to domains II and III of Pseudomonas exotoxin A (28). This fusion protein was reported to specifically bind and inhibit protein synthesis
15 in cells expressing the HIV envelope glycoprotein gp120 (25).

We have now discovered that a specific CD4-gamma1 chimeric heavy chain homodimer provides advantages relative to those
20 CD4-IgG1 heavy chain homodimers which have been described more than one year ago. Specifically, we have constructed a CD4-gamma1 chimeric heavy chain homodimer which contains the V1V2 domains of CD4 and which is efficiently assembled intracellularly and efficiently secreted from mammalian
25 cells as a homodimer, enabling high recovery and purification from the medium of cells expressing this chimeric heavy chain homodimer. To construct this homodimer, we have used the entire hinge, CH2, and CH3 domains from a human gamma1 heavy chain, which results in a
30 chimeric molecule containing the constant domains of a human IgG1 molecule responsible for dimerization and efficient secretion. This is in contrast to the heavy chain dimers described by Capon and Gregory (20) which include the CH1 domain in the CD4-IgG1 heavy chain dimer, resulting in poor
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secretion and recovery from cell culture medium of the recombinant molecule. We have also included the entire hinge domain of gamma1 heavy chain in the CD4-gamma1 chimeric heavy chain homodimer of this invention to provide efficient dimerization, since the cysteine residues contained in this domain are responsible for forming the disulphide links to the second chain of the homodimer, positioning the two chains in the correct spatial alignment and facilitating formation of the antigen combining site.

Furthermore, by including the entire hinge domain, we have maintained the segmental flexibility of the heavy chain dimers, thus enabling modulation of biological function such as complement activation and Fc receptor binding (29).

In addition to the CD4-gamma1 chimeric heavy chain homodimers, we have also constructed CD4-IgG1 heavy chains, which contain the V1V2 domains of CD4 fused to the CH1, hinge, CH2 and CH3 domains of human gamma1 heavy chain. These molecules encode a CD4-IgG1 chimeric heterotetramer and, when co-expressed in the presence of CD4-kappa chimeric light chains containing the V1 and V2 domains of CD4 fused to the entire constant domain of human kappa light chains (or lambda light chains), enable the production of said heterotetramer. This heterotetramer comprises two CD4-IgG1 chimeric heavy chains and two CD4-kappa chimeric light chains. Producing heavy chains which contain the CH1 domain enables efficient association with the CD4-kappa chimeric light chains, resulting in efficient secretion of a CD4-IgG1 chimeric heterotetramer. These CD4-IgG1 chimeric heterotetramers possess increased serum half-lives and increased avidity for HIV as compared with heavy chain dimers.

Summary of the Invention

This invention provides an expression vector encoding a CD4-
gammal chimeric heavy chain homodimer. This invention also
5 provides an expression vector encoding the heavy chains of
a CD4-IgG1 chimeric heterotetramer. Finally, this invention
provides an expression vector encoding the light chains of
a CD4-IgG1 chimeric heterotetramer.

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Brief Description of the Figures

5 Figure 1: A) Domain structure of CD4-gamma1 chimeric heavy chain gene; B) Protein structure of CD4-gamma1 chimeric heavy chain homodimer. The sequence shown below is the single letter amino acid code of the junction between CD4 (phe179) and the hinge region of human gamma1 heavy chain. Note that the hinge region of a gamma1 heavy chain contains three cysteines (see text for discussion). Abbreviations: L, leader (signal) sequence of human CD4; V1V2, amino-terminal variable-like domains of human CD4; H, hinge region of human gamma1 heavy chain; CH2 and CH3, second and third constant regions of human gamma1 heavy chain.

15 Figure 2: A) Domain structure of chimeric genes used to express CD4-IgG1 chimeric heterotetramer. Top, CD4-gamma1 chimeric heavy chain gene; Bottom, CD4-kappa chimeric light chain gene. B) Protein structure of CD4-IgG1 chimeric heterotetramer. Abbreviations: CH1-CH2-CH3, first, second and third constant regions of human gamma1 heavy chain; C-kappa, constant region of human kappa light chain.

25 Figure 3: DNA and predicted protein sequence of a CD4-gamma1 chimeric heavy chain homodimer (one chain). The numbers at the end of each line indicate the nucleotide positions. The numbers above each line indicate the amino acid positions (given in single letter code). The protein domains are indicated above the sequences by arrows.

30 Figure 4: DNA and predicted protein sequence of a CD4-IgG1 chimeric heavy chain of the CD4-IgG1 chimeric heterotetramer. The numbers at the end of each line indicate the nucleotide positions. The numbers above each line indicate the amino acid positions (given in single

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letter code). The protein domains are indicated above the sequences by arrows.

5 Figure 5: DNA and predicted protein sequence of a CD4-kappa chimeric light chain of the CD4-IgG1 chimeric heterotetramer. The numbers at the end of each line indicate the nucleotide positions. The numbers above each line indicate the amino acid positions (given in single letter code). The protein domains are indicated above the sequences by arrows.

10 Figure 6: Secretion of CD4-gamma1 chimeric heavy chain homodimer from transfected cells. Cos-M5 cells were mock transfected, transfected with CD4-IgG1-pcDNA1 DNA, or transfected with CD4-gamma2 chimeric heavy chain mammalian expression vector DNA. At 48-72 hours post-transfection,
15 the cells were radiolabelled with ³⁵S-methionine. Radiolabelled medium was precipitated with Protein-A sepharose beads. The precipitated proteins were analyzed by SDS-PAGE under reducing or non-reducing conditions and were
20 visualized by fluorography. Lane M, medium from mock transfected cells; Lane 1, medium from cells transfected with CD4-IgG1-pcDNA1 DNA; Lane 2, medium from cells transfected with CD4-gamma2 chimeric heavy chain mammalian expression vector DNA.

25 Figure 7: Precipitation of HIV-1 gp120 with CD4-gamma1 chimeric heavy chain homodimer. Cos-M5 cells were mock transfected, transfected with the CD4-IgG1-pcDNA1, or transfected with CD4-gamma2 chimeric heavy chain mammalian expression vector DNA. At 48-72 hours post transfection,
30 unlabelled aliquots of medium were incubated with an aliquot of ³⁵S-methionine-labelled gp120. The complexes were precipitated with Protein A-sepharose beads. The precipitates were then analyzed by SDS-PAGE followed by fluorography. Lane M, medium from mock transfected cells;
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Lane 1, medium from cells transfected with CD4-IgG1-pcDNA1 DNA; Lane 2, medium from cells transfected with CD4-gamma2 chimeric heavy chain mammalian expression vector DNA.

5 Figure 8: Purification of CD4-gamma1 chimeric heavy chain homodimer from CHO cell-conditioned medium. Stable CHO cells constitutively secreting CD4-gamma1 chimeric heavy chain homodimer, or CD4-gamma2 chimeric heavy chain homodimer, were grown in roller bottles. Conditioned medium was passed over a Protein A-sepharose column and bound material was eluted from the column. The peak fractions were identified by SDS-PAGE followed by silver staining and pooled. The purified proteins were then analyzed by SDS-PAGE under reducing conditions followed by silver staining. Lane 1, CD4-gamma1 chimeric heavy chain homodimer; Lane 2, CD4-gamma2 chimeric heavy chain homodimer.

15 Figure 9: Inhibition of HIV binding to CEM cells by CD4-based molecules. Soluble CD4 (sCD4), partially purified CD4-gamma1, or partially purified CD4-gamma2 were tested for inhibition of virus binding to CD4 positive cells. Bound virus was detected by indirect immunofluorescence and cytofluorography. Results are expressed as percent inhibition versus concentration of inhibiting agent.

25 Figure 10: Inhibition of HIV infection of CD4-positive cells by CD4-based molecules. sCD4, partially purified CD4-gamma1, or partially purified CD4-gamma2 were incubated with an HIV-1 inoculum (100 TCID₅₀), and mixtures were added to PHA-stimulated lymphocytes and incubated at 37°C overnight. The cells were washed and plated in microculture (1 x 10⁵ cells/culture; 10 cultures per dilution) and monitored for reproductive viral replication by detection of HIV antigen in culture supernates 8 and 12 days later. Results are expressed as percent positive cultures at a given concentration of inhibiting agent.

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Figure 11: Purification of CD4-gammal chimeric heavy chain homodimer. Stable CHO cells constitutively secreting CD4-gammal chimeric heavy chain homodimer were grown in roller bottles. Conditioned medium was passed over a Protein A-sepharose column and bound material was eluted from the column (see Figure 8). The peak fractions were then pooled and passed over an S-sepharose column. After extensive washes, the CD4-gammal chimeric heavy chain homodimer was eluted with 50mM BES pH 7.0, 500mM NaCl. The peak fractions were identified by SDS-PAGE followed by silver staining and pooled. The purified protein was then analyzed by SDS-PAGE under non-reducing and reducing conditions followed by silver staining. Lane 1: approximately 1.5 μ g protein run under non-reducing conditions, Lane 2: approximately 1.5 μ g protein run under reducing conditions.

Figure 12: Secretion of CD4-IgG1 chimeric heterotetramer from stably transfected cells. CHO cells stably expressing both CD4-IgG1 chimeric heavy chains and CD4-kappa chimeric light chains were radiolabelled with ³⁵S-methionine and cysteine. Radiolabelled medium was precipitated with Protein-A sepharose beads. (A) The precipitated proteins were analyzed by SDS-PAGE under non-reducing conditions, and were visualized by fluorography. Lane 1: medium from untransfected CHO cells, Lane 2: medium from cells stably expressing both the CD4-IgG1 chimeric heavy chains, and CD4-kappa chimeric light chains. (B) An identical sample to that run in lane 2 from (A) was run on SDS-PAGE under non-reducing conditions. The lane from this SDS-PAGE gel was excised and the proteins reduced by incubation of the gel slice for 45 minutes at 4°C in equilibration buffer (62.5 mM TrisHCl pH 6.8, 2.3% SDS, 5% β -mercaptoethanol, 10% glycerol). After incubation of the gel slice under reducing conditions, the proteins contained within the gel were analyzed by SDS-PAGE and visualized by fluorography.

Detailed Description of the Invention

Five expression vectors and two plasmids designated CD4-IgG2-Rf, CD4-IgG1-Rf, CD4-IgG1HC-pRcCMV, CD4-IgG2HC-pRcCMV, CD4-kLC-pRcCMV, CD4-IgG1-pcDNA1, and CD4-IgG2-pcDNA, respectively have been deposited with the American Typ Culture Collection, Rockville, Maryland, U.S.A. 20852, under ATCC Accession No. 40949, 40950, 75192, 75193, 75194, 40951, and 40952, respectively. These deposits were made pursuant to the provisions of the Budapest Treaty on the International Recognition of the Deposit of Microorganisms for the Purposes of Patent Procedure (Budapest Treaty).

Specifically, the invention provides an expression vector designated CD4-IgG1-pcDNA1 (ATCC No. 40951) encoding a CD4-gammal chimeric heavy chain homodimer. The invention additionally provides a CD4-gammal chimeric heavy chain homodimer encoded by this expression vector or any other expression vector having the same DNA coding region inserted therein. Specifically, the invention also provides expression vectors designated CD4-IgG1HC-pRcCMV and CD4-kLC-pRcCMV (ATCC Nos. 75192 and 75194), encoding a CD4-IgG1 chimeric heavy chain and a CD4-kappa chimeric light chain. The invention additionally provides a CD4-IgG1 chimeric heterotetramer encoded by these expression vectors or any other expression vector having the same DNA encoding region inserted therein.

In accordance with the invention, numerous vector systems for expression may be employed. For example, one class of vectors utilizes DNA elements which are derived from animal viruses such as bovine papilloma virus, polyoma virus, adenovirus, vaccinia virus, baculovirus, retroviruses (RSV, MMTV or MOMLV) or SV40 virus. Additionally, cells which have stably integrated the DNA into their chromosomes may be

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histocompatibility complex (MHC) class II molecules on the surface of antigen-presenting cells to mediate efficient cellular immune response interactions. In man, CD4 is also the target of interaction with the human immunodeficiency virus (HIV).

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developments combining the techniques of molecular genetics with monoclonal antibody technology has lead to the production of "humanized" chimeric antibodies in vitro. In these chimeric antibodies, the variable domains of human immunoglobulin heavy and light chains are replaced with specific heavy and light chain variable domains from a murine monoclonal antibody (17-19). The result of this genetic manipulation is a molecule with specificity for a particular antigen and the characteristics of human immunoglobulins.

Sequence and structural analyses of CD4 indicate that the four extracellular domains are immunoglobulin-like. Since the Fc portion of immunoglobulins controls the rate of catabolism of the molecules (serum half-life ranging from 14 to 21 days) and provides various effector functions, several reports describe the replacement of variable and constant domains of immunoglobulins with the immunoglobulin-like domains of CD4 (21-24).

CD4-IgG1 heavy chain fusion proteins resulting in chimeric gammal heavy chain dimers have been described (21). These molecules contain the gammal heavy chain CH1 domain in addition to the hinge, CH2 and CH3 domains. However, heavy chain assembly and secretion from mammalian cells is less efficient if the CH1 domain is expressed in the absence of light chains (25). Subsequently, a CD4-IgG1 heavy chain fusion protein lacking the CH1 domain and the first five amino acids of the hinge region was described which was secreted to high levels (22). These fusion proteins retain various effector functions of immunoglobulin molecules, such as Fc receptor binding, antibody-dependent cell-mediated cytotoxicity (ADCC) toward HIV-1-infected cells, and placental transfer via an Fc receptor-dependent mechanism (22). CD4-IgM heavy chain fusion proteins have also been described (26). In addition, CD4-IgG1 fusion proteins have

been described wherein the V1V2 domains of CD4 are fused to the CH1, hinge, CH2 and CH3 domains of a gamma1 heavy chain, and wherein the V1V2 domains of CD4 are fused to the constant domain of a kappa light chain (29).

5 Fusion proteins linking CD4 to toxins have also been constructed and tested for their ability to kill HIV-infected cells. In one study, sCD4 was coupled to the deglycosylated A chain of ricin which inactivates ribosomes, therefore inhibiting protein synthesis and killing the cell (27). This fusion protein was reported to specifically lyse
10 cells infected with five different isolates of HIV, but was nontoxic to uninfected cells. In another study, the V1V2 domains of CD4 were coupled to domains II and III of *Pseudomonas* exotoxin A (28). This fusion protein was reported to specifically bind and inhibit protein synthesis
15 in cells expressing the HIV envelope glycoprotein gp120 (25).

We have now discovered that a specific CD4-gamma1 chimeric heavy chain homodimer provides advantages relative to those
20 CD4-IgG1 heavy chain homodimers which have been described more than one year ago. Specifically, we have constructed a CD4-gamma1 chimeric heavy chain homodimer which contains the V1V2 domains of CD4 and which is efficiently assembled intracellularly and efficiently secreted from mammalian
25 cells as a homodimer, enabling high recovery and purification from the medium of cells expressing this chimeric heavy chain homodimer. To construct this homodimer, we have used the entire hinge, CH2, and CH3 domains from a human gamma1 heavy chain, which results in a
30 chimeric molecule containing the constant domains of a human IgG1 molecule responsible for dimerization and efficient secretion. This is in contrast to the heavy chain dimers described by Capon and Gregory (20) which include the CH1 domain in the CD4-IgG1 heavy chain dimer, resulting in poor
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secretion and recovery from cell culture medium of the recombinant molecule. We have also included the entire hinge domain of gamma1 heavy chain in the CD4-gamma1 chimeric heavy chain homodimer of this invention to provide efficient dimerization, since the cysteine residues contained in this domain are responsible for forming the disulphide links to the second chain of the homodimer, positioning the two chains in the correct spatial alignment and facilitating formation of the antigen combining site.

Furthermore, by including the entire hinge domain, we have maintained the segmental flexibility of the heavy chain dimers, thus enabling modulation of biological function such as complement activation and Fc receptor binding (29).

In addition to the CD4-gamma1 chimeric heavy chain homodimers, we have also constructed CD4-IgG1 heavy chains, which contain the V1V2 domains of CD4 fused to the CH1, hinge, CH2 and CH3 domains of human gamma1 heavy chain. These molecules encode a CD4-IgG1 chimeric heterotetramer and, when co-expressed in the presence of CD4-kappa chimeric light chains containing the V1 and V2 domains of CD4 fused to the entire constant domain of human kappa light chains (or lambda light chains), enable the production of said heterotetramer. This heterotetramer comprises two CD4-IgG1 chimeric heavy chains and two CD4-kappa chimeric light chains. Producing heavy chains which contain the CH1 domain enables efficient association with the CD4-kappa chimeric light chains, resulting in efficient secretion of a CD4-IgG1 chimeric heterotetramer. These CD4-IgG1 chimeric heterotetramers possess increased serum half-lives and increased avidity for HIV as compared with heavy chain dimers.

Summary of the Invention

This invention provides an expression vector encoding a CD4-gamma1 chimeric heavy chain homodimer. This invention also provides an expression vector encoding the heavy chains of a CD4-IgG1 chimeric heterotetramer. Finally, this invention provides an expression vector encoding the light chains of a CD4-IgG1 chimeric heterotetramer.

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Brief Description of the Figures

5 **Figure 1:** A) Domain structure of CD4-gamma1 chimeric heavy chain gene; B) Protein structure of CD4-gamma1 chimeric heavy chain homodimer. The sequence shown below is the single letter amino acid code of the junction between CD4 (phe179) and the hinge region of human gamma1 heavy chain. Note that the hinge region of a gamma1 heavy chain contains three cysteines (see text for discussion). Abbreviations:
10 L, leader (signal) sequence of human CD4; V1V2, amino-terminal variable-like domains of human CD4; H, hinge region of human gamma1 heavy chain; CH2 and CH3, second and third constant regions of human gamma1 heavy chain.

15 **Figure 2:** A) Domain structure of chimeric genes used to express CD4-IgG1 chimeric heterotetramer. Top, CD4-gamma1 chimeric heavy chain gene; Bottom, CD4-kappa chimeric light chain gene. B) Protein structure of CD4-IgG1 chimeric heterotetramer. Abbreviations: CH1-CH2-CH3, first, second and third constant regions of human gamma1 heavy chain; C-
20 kappa, constant region of human kappa light chain.

25 **Figure 3:** DNA and predicted protein sequence of a CD4-gamma1 chimeric heavy chain homodimer (one chain). The numbers at the end of each line indicate the nucleotide positions. The numbers above each line indicate the amino acid positions (given in single letter code). The protein domains are indicated above the sequences by arrows.

30 **Figure 4:** DNA and predicted protein sequence of a CD4-IgG1 chimeric heavy chain of the CD4-IgG1 chimeric heterotetramer. The numbers at the end of each line indicate the nucleotide positions. The numbers above each line indicate the amino acid positions (given in single

letter code). The protein domains are indicated above the sequences by arrows.

Figure 5: DNA and predicted protein sequence of a CD4-kappa chimeric light chain of the CD4-IgG1 chimeric heterotetramer. The numbers at the end of each line indicate the nucleotide positions. The numbers above each line indicate the amino acid positions (given in single letter code). The protein domains are indicated above the sequences by arrows.

Figure 6: Secretion of CD4-gamma1 chimeric heavy chain homodimer from transfected cells. Cos-M5 cells were mock transfected, transfected with CD4-IgG1-pcDNA1 DNA, or transfected with CD4-gamma2 chimeric heavy chain mammalian expression vector DNA. At 48-72 hours post-transfection, the cells were radiolabelled with ³⁵S-methionine. Radiolabelled medium was precipitated with Protein-A sepharose beads. The precipitated proteins were analyzed by SDS-PAGE under reducing or non-reducing conditions and were visualized by fluorography. Lane M, medium from mock transfected cells; Lane 1, medium from cells transfected with CD4-IgG1-pcDNA1 DNA; Lane 2, medium from cells transfected with CD4-gamma2 chimeric heavy chain mammalian expression vector DNA.

Figure 7: Precipitation of HIV-1 gp120 with CD4-gamma1 chimeric heavy chain homodimer. Cos-M5 cells were mock transfected, transfected with the CD4-IgG1-pcDNA1, or transfected with CD4-gamma2 chimeric heavy chain mammalian expression vector DNA. At 48-72 hours post transfection, unlabelled aliquots of medium were incubated with an aliquot of ³⁵S-methionine-labelled gp120. The complexes were precipitated with Protein A-sepharose beads. The precipitates were then analyzed by SDS-PAGE followed by fluorography. Lane M, medium from mock transfected cells;

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Lane 1, medium from cells transfected with CD4-IgG1-pcDNA1 DNA; Lane 2, medium from cells transfected with CD4-gamma2 chimeric heavy chain mammalian expression vector DNA.

5 Figure 8: Purification of CD4-gamma1 chimeric heavy chain homodimer from CHO cell-conditioned medium. Stable CHO cells constitutively secreting CD4-gamma1 chimeric heavy chain homodimer, or CD4-gamma2 chimeric heavy chain homodimer, were grown in roller bottles. Conditioned medium was passed over a Protein A-sepharose column and bound material was eluted from the column. The peak fractions were identified by SDS-PAGE followed by silver staining and pooled. The purified proteins were then analyzed by SDS-PAGE under reducing conditions followed by silver staining. Lane 1, CD4-gamma1 chimeric heavy chain homodimer; Lane 2, CD4-gamma2 chimeric heavy chain homodimer.

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Figure 9: Inhibition of HIV binding to CEM cells by CD4-based molecules. Soluble CD4 (sCD4), partially purified CD4-gamma1, or partially purified CD4-gamma2 were tested for inhibition of virus binding to CD4 positive cells. Bound virus was detected by indirect immunofluorescence and cytofluorography. Results are expressed as percent inhibition versus concentration of inhibiting agent.

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Figure 10: Inhibition of HIV infection of CD4-positive cells by CD4-based molecules. sCD4, partially purified CD4-gamma1, or partially purified CD4-gamma2 were incubated with an HIV-1 inoculum (100 TCID₅₀), and mixtures were added to PHA-stimulated lymphocytes and incubated at 37°C overnight. The cells were washed and plated in microculture (1 x 10⁵ cells/culture; 10 cultures per dilution) and monitored for reproductive viral replication by detection of HIV antigen in culture supernates 8 and 12 days later. Results are expressed as percent positive cultures at a given concentration of inhibiting agent.

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Figure 11: Purification of CD4-gammal chimeric heavy chain homodimer. Stable CHO cells constitutively secreting CD4-gammal chimeric heavy chain homodimer were grown in roller bottles. Conditioned medium was passed over a Protein A-sepharose column and bound material was eluted from the column (see Figure 8). The peak fractions were then pooled and passed over an S-sepharose column. After extensive washes, the CD4-gammal chimeric heavy chain homodimer was eluted with 50mM BES pH 7.0, 500mM NaCl. The peak fractions were identified by SDS-PAGE followed by silver staining and pooled. The purified protein was then analyzed by SDS-PAGE under non-reducing and reducing conditions followed by silver staining. Lane 1: approximately 1.5 μ g protein run under non-reducing conditions, Lane 2: approximately 1.5 μ g protein run under reducing conditions.

Figure 12: Secretion of CD4-IgG1 chimeric heterotetramer from stably transfected cells. CHO cells stably expressing both CD4-IgG1 chimeric heavy chains and CD4-kappa chimeric light chains were radiolabelled with ³⁵S-methionine and cysteine. Radiolabelled medium was precipitated with Protein-A sepharose beads. (A) The precipitated proteins were analyzed by SDS-PAGE under non-reducing conditions, and were visualized by fluorography. Lane 1: medium from untransfected CHO cells, Lane 2: medium from cells stably expressing both the CD4-IgG1 chimeric heavy chains, and CD4-kappa chimeric light chains. (B) An identical sample to that run in lane 2 from (A) was run on SDS-PAGE under non-reducing conditions. The lane from this SDS-PAGE gel was excised and the proteins reduced by incubation of the gel slice for 45 minutes at 4°C in equilibration buffer (62.5 mM TrisHCl pH 6.8, 2.3% SDS, 5% β -mercaptoethanol, 10% glycerol). After incubation of the gel slice under reducing conditions, the proteins contained within the gel were analyzed by SDS-PAGE and visualized by fluorography.

Detailed Description of the Invention

Five expression vectors and two plasmids designated CD4-IgG2-Rf, CD4-IgG1-Rf, CD4-IgG1HC-pRcCMV, CD4-IgG2HC-pRcCMV, CD4-kLC-pRcCMV, CD4-IgG1-pcDNA1, and CD4-IgG2-pcDNA, respectively have been deposited with the American Type Culture Collection, Rockville, Maryland, U.S.A. 20852, under ATCC Accession No. 40949, 40950, 75192, 75193, 75194, 40951, and 40952, respectively. These deposits were made pursuant to the provisions of the Budapest Treaty on the International Recognition of the Deposit of Microorganisms for the Purposes of Patent Procedure (Budapest Treaty).

Specifically, the invention provides an expression vector designated CD4-IgG1-pcDNA1 (ATCC No. 40951) encoding a CD4-gamma1 chimeric heavy chain homodimer. The invention additionally provides a CD4-gamma1 chimeric heavy chain homodimer encoded by this expression vector or any other expression vector having the same DNA coding region inserted therein. Specifically, the invention also provides expression vectors designated CD4-IgG1HC-pRcCMV and CD4-kLC-pRcCMV (ATCC Nos. 75192 and 75194), encoding a CD4-IgG1 chimeric heavy chain and a CD4-kappa chimeric light chain. The invention additionally provides a CD4-IgG1 chimeric heterotetramer encoded by these expression vectors or any other expression vector having the same DNA encoding region inserted therein.

In accordance with the invention, numerous vector systems for expression may be employed. For example, one class of vectors utilizes DNA elements which are derived from animal viruses such as bovine papilloma virus, polyoma virus, adenovirus, vaccinia virus, baculovirus, retroviruses (RSV, MMTV or MOMLV) or SV40 virus. Additionally, cells which have stably integrated the DNA into their chromosomes may be

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5 selected by introducing one or more markers which allow selection of transfected host cells. The marker may provide for prototrophy to an auxotrophic host, biocide resistance, e.g., antibiotics, or resistance to heavy metals such as copper or the like. The selectable marker gene can be either directly linked to the DNA sequences to be expressed, or introduced into the same cell by cotransformation. Additional elements may also be needed for optimal synthesis of mRNA. These elements may include splice signals, as well as transcriptional promoters, enhancers, and termination signals. The cDNA expression vectors incorporating such elements include those described by Okayama. (32)

10 Thus, the invention further provides a method of producing a CD4-gammal chimeric heavy chain homodimer. This method comprises

- 15
- a) transfecting a mammalian cell with an expression vector for producing the CD4-gammal chimeric heavy chain homodimer;
 - 20 b) culturing the resulting transfected mammalian cell under conditions such that CD4-gammal chimeric heavy chain homodimer is produced; and
 - c) recovering the CD4-gammal chimeric heavy chain homodimer so produced.

25 Once the vector or DNA sequence containing the constructs has been prepared for expression, the expression vectors may be transfected or introduced into an appropriate mammalian cell host. Various techniques may be employed such as protoplast fusion, calcium phosphate precipitation, 30 electroporation or other conventional techniques. In the case of protoplast fusion, the cells are grown in media and screened for the appropriate activity. Expression of the gene(s) results in production of the fusion protein which corresponds to one chain of the CD4-gammal chimeric heavy

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chain homodimer. This fusion protein may then be treated to form the chimeric heavy chain homodimer.

Further, methods and conditions for culturing the resulting transfected cells and for recovering the chimeric heavy chain homodimer so produced are well known to those skilled in the art and may be varied or optimized depending upon the specific expression vector and mammalian host cell employed.

In accordance with the claimed invention, the preferred host cells for expressing the chimeric heavy chain homodimers of this invention are mammalian cell lines, including, for example, monkey kidney CV1 line transformed by SV40 (COS-7); human embryonic kidney line 293; baby hamster kidney cells (BHK); Chinese hamster ovary-cells-DHFR (CHO); monkey kidney cells (CV1); African green monkey kidney cells (VERO-76); human cervical carcinoma cells (HELA); canine kidney cells (MDCK); human lung cells (W138); human liver cells (Hep G2); mouse mammary tumor (MMT 060562); mouse cell line (C127) and myeloma cell lines.

The invention further provides a method of inhibiting the HIV infection of a CD4+ cell which comprises treating the CD4+ cell with the CD4-gammal chimeric heavy chain homodimer in an amount which is effective to inhibit infection of the cell.

Additionally, the invention provides a method of preventing a subject from being infected with HIV which comprises administering to the subject the CD4-gammal chimeric heavy chain homodimer in an amount which is effective to prevent the subject from being infected with HIV.

Although the invention encompasses the administration of the chimeric heavy chain homodimer to various subjects, AIDS patients are of particular interest. Further, methods of

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administering the homodimer are well known in the art and include, merely by way of example, subcutaneous, intramuscular and intravascular injection, alone or in combination with other agents such as AZT or DDI.

5 Further provided is a method of treating a subject infected with HIV so as to block the spread of HIV infection which comprises administering to the subject an amount of the CD4-gammal chimeric heavy chain homodimer in an amount which is effective to block the spread of HIV infection.

10 For example, the homodimer may be administered to patients having HIV infection at a dosage capable of maintaining a concentration of greater than about 100 ng of CD4-gammal chimeric heavy chain homodimer/ml plasma. For CD4-gammal chimeric heavy chain homodimer variants having different
15 molecular weights, about 2 picomoles of soluble receptor per ml of plasma, an amount for example, sufficient to establish a stoichiometric equivalence with native (membrane bound) and soluble receptor is administered. Typically, the dosage of soluble CD4 is about 100 μ g/kg of patient weight/day.
20

The foregoing method may be used to help prevent the spread of the HIV virus within the body of a HIV infected patient. Additionally, CD4-gammal chimeric heavy chain homodimer may be administered as a prophylactic measure to render a
25 subject's blood less susceptible to the spread of the HIV virus. Such prophylactic administration includes administration both prior to HIV contact or shortly thereafter, or both.

30 A pharmaceutical composition which comprises the CD4-gammal chimeric heavy chain homodimer of this invention in an amount effective to inhibit HIV infection of a CD4+ cell and a pharmaceutically acceptable carrier is further provided.
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Pharmaceutically acceptable carriers are well known in the art to which the present invention pertains and include, but are not limited to, 0.01-0.1M and preferably 0.05 M phosphate buffer or 0.8% saline. Additionally, such pharmaceutically acceptable carriers may be aqueous or non-
5 non-aqueous solutions, suspensions, and emulsions. Examples of non-aqueous solvents are propylene glycol, polyethylene glycol, vegetable oils such as olive oil, and injectable organic esters such as ethyl oleate. Aqueous carriers include water, alcoholic/aqueous solutions, emulsions or
10 suspensions, including saline and buffered media. Parenteral vehicles include sodium chloride solution, Ringer's dextrose, dextrose and sodium chloride, lactated Ringer's or fixed oils. Intravenous vehicles include fluid and nutrient replenishers, electrolyte replenishers such as those based on Ringer's dextrose, and the like.
15 Preservatives and other additives may also be present, such as, for example, antimicrobials, antioxidants, chelating agents, inert gases and the like. (33)

20 The invention further provides a composition of matter comprising a CD4-gammal chimeric heavy chain homodimer and a toxin linked thereto.

Some example of toxins are the deglycosylated A chain of ricin, domains II or III of Pseudomonas exotoxin A, Diphtheria toxin, or a non-peptidyl cytotoxin. These toxins
25 may be linked using conventional in vitro protein cross-linking agents (34-36). Additionally the toxins may be linked by recombinant synthesis as a fusion protein (see for example U.S. Patent 4,765,382).

30 The invention also provides a diagnostic reagent comprising a CD4-IgG1 chimeric heavy chain homodimer and a detectable marker linked thereto. By employing a molecule which binds to the HIV virus and additionally has attached to it a
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detectable marker, one may identify cells which are infected with HIV. Examples of conventional detectable markers includes radioisotopes such as I125, chromophores, and fluorophores.

5 Thus, the chimeric heavy chain homodimer of the invention may be used in an assay for HIV or SIV viral infection in a biological sample by contacting a sample derived from an animal suspected of having an HIV or SIV infection, with the homodimer of the invention, and detecting whether a complex
10 forms with gp120, either alone or on the surface of an HIV-infected cell. For this purpose the homodimer may be labeled with a detectable marker or may be unlabeled and then be detected with another reagent which is detectably labeled and is specifically directed to the homodimer or to
15 a complex between it and gp120.

For example, a biological sample may be treated with nitro-cellulose, or another solid support which is capable of immobilizing cells, cell particles or soluble protein. The support may then be washed with suitable buffers followed by
20 treatment with the chimeric heavy chain homodimer which may be detectably labeled. The solid phase support may then be washed with buffer a second time to remove unbound fusion protein and the labeled homodimer detected.

25 In carrying out the assay the following steps may be employed.

- 30 a) contacting a sample suspected of containing gp120 with a solid support to effect immobilization of gp120, or cells which express gp120 on their surface;
- b) contacting said solid support with the detectably labeled chimeric heavy chain homodimer of the invention;

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- c) incubating said detectably labeled homodimer with said support for a sufficient amount of time to allow the homodimer to bind to the immobilized gp120 or cell which expresses gp120 on its surface;
 - d) separating the solid phase support from the incubation mixture obtained in step c); and
 - e) detecting bound labeled homodimer and thereby detecting gp120.

10 Such a method may be formatted either as a qualitative or as a quantitative test using methods well known in the art.

15 Alternatively, labeled homodimer-gp120 complex may be separated from a reaction mixture by contacting the complex with an immobilized antibody or protein which is specific for an immunoglobulin or, e.g., protein A, protein G, or anti-IgG antibodies. Such anti-immunoglobulin antibodies may be monoclonal or polyclonal. The solid support may then be washed with suitable buffers to obtain an immobilized gp120-labeled homodimer-antibody complex. The label on the homodimer may then be detected so as to measure endogenous gp120, and thereby detect the presence of HIV.

20 In one embodiment of the invention, a method for detecting HIV or SIV viral infection in a sample is provided comprising:

- 25
- a) contacting a sample suspected of containing gp120 with a CD4-gamma1 chimeric heavy chain homodimer in accordance with this invention, and the Fc portion of an immunoglobulin chain; and
 - 30 b) detecting whether a complex is formed.

The invention also provides a method of detecting gp120 in a sample comprising:

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- 5 a) contacting a mixture obtained by contacting a sample suspected of containing gp120 with a homodimer of this invention, and the Fc portion of an immunoglobulin chain, with an Fc binding molecule, such as an antibody, protein A, or protein G, which is immobilized on a solid phase support and is specific for the homodimer, to obtain a gp120-homodimer immobilized antibody complex,
- 10 b) washing the solid phase support obtained in step (a) to remove unbound homodimer; and
- c) detecting the homodimer.

15 Of course, the specific concentrations of unlabeled or detectably labeled homodimer and gp120, the temperature and time of incubation, as well as other assay conditions, may be varied depending on various factors including the concentration of gp120 in the sample, the nature of the sample, and the like. Those skilled in the art are readily able to determine operative and optimal assay conditions for each determination.

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Also provided is an enzyme-linked immunoadsorbent assay (ELISA) to detect and quantify soluble CD4 (sCD4) or CD4 chimeric proteins. In carrying out the assay, the process comprises:

- 25 a) contacting a sample containing sCD4 with a solid support to immobilize soluble sCD4;
- b) contacting said solid support with the detectably labeled monoclonal antibody OKT4a alone, or with a sample containing sCD4 or CD4 chimeric proteins and
- 30 OKT4a;
- c) incubating said detectably labeled OKT4a containing media for sufficient time to allow for binding to immobilized sCD4;
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- d) separating the solid phase support from the incubation mixture in step (c);
- e) detecting the bound OKT4a and thereby quantifying the amount of CD4 contained in the sample.

5 The invention further provides an expression vector encoding the heavy chains of a CD4-IgG1 chimeric heterotetramer, designated CD4-IgG1HC-pRcCMV (ATCC No. 75192). The invention also provides a CD4-IgG1 chimeric heterotetramer, the heavy chains of which are encoded by this expression
10 vector or another vector containing the same coding sequence.

Additionally, the invention provides an expression vector encoding the light chains of a CD4-IgG1 chimeric heterotetramer, designated CD4-kLC-pRcCMV (ATCC No. 75194).
15 Finally, the invention provides a CD4-IgG1 chimeric heterotetramer, the light chains of which are encoded by the CD4-kLC-pRcCMV expression vector or another vector containing the same coding sequence.

20 Further, the invention provides a CD4-IgG1 chimeric heterotetramer both the heavy and light chains of which are encoded by the aforementioned expression vectors.

25 The invention further provides a method of producing such a CD4-IgG1 chimeric heterotetramer. This method comprises:

- a) cotransfecting a mammalian cell with the expression vector for producing the light chains of a CD4-IgG1 chimeric heterotetramer and an expression vector encoding a light chain;
 - 30 b) culturing the resulting cotransfected mammalian cell under conditions such that CD4-IgG1 chimeric heterotetramer is produced; and
 - c) recovering the CD4-IgG1 chimeric heterotetramer so produced.
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Methods of cotransfecting mammalian cells are well known in the art and include those discussed hereinabove. Similarly, expression vectors encoding light chains are well known in the art.

5 The invention additionally provides a method of producing a CD4-IgG1 chimeric heterotetramer which comprises:

- 10 a) cotransfecting a mammalian cell with the expression vector for producing the light chains of a CD4-IgG1 chimeric heterotetramer and with an expression vector encoding an IgG1 heavy chain;
- b) culturing the resulting cotransfected mammalian cell under conditions such that a CD4-IgG1 chimeric hetero-tetramer is produced; and
- 15 c) recovering the CD4-IgG1 chimeric heterotetramer so produced.

Further the invention provides a method of producing an CD4-IgG1 chimeric heterotetramer which comprises:

- 20 a) cotransfecting a mammalian cell with the expression vector for producing the heavy chains of a CD4-IgG1 chimeric heterotetramer and an expression vector for producing the light chains of a CD4-IgG1 chimeric heterotetramer;
- b) culturing the resulting cotransfected mammalian cell under conditions such that the CD4-IgG1 chimeric heterotetramer is produced; and
- 25 c) recovering the CD4-IgG1 chimeric heterotetramer so produced.

30 The invention also includes a method of inhibiting HIV infection of a CD4+ cell which comprises treating the CD4+ cell with either a CD4-IgG1 chimeric heterotetramer, the heavy chains of which are encoded by the expression vector designated CD4-IgG1HC-pRcCMV; a CD4-IgG1 chimeric heterotetramer, the light chains of which are encoded by the

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expression vector designated CD4-kLC-pRCCMV; or a CD4-IgG1 chimeric heterotetramer, both the heavy and the light chains of which are encoded by both of the above expression vectors, in an amount effective to inhibit infection of the cell.

5 The invention further provides a method of preventing a subject from being infected with HIV. This method comprises administering to the subject either a CD4-IgG1 chimeric heterotetramer, the heavy chains of which are encoded by the
10 expression vector designated CD4-IgG1HC-pRCCMV; a CD4-IgG1 chimeric heterotetramer, the light chains of which are encoded by the expression vector designated CD4-kLC-pRCCMV; or a CD4-IgG1 chimeric heterotetramer, both the heavy and the light chains of which are encoded by the above expression vectors, in an amount which is effective to
15 prevent the subject from being infected with HIV.

The invention also provides a method of treating a subject infected with HIV so as to block the spread of HIV infection. This method comprises administering to the
20 subject either a CD4-IgG1 chimeric heterotetramer, the heavy chains of which are encoded by the expression vector designated CD4-IgG1HC-pRCCMV; a CD4-IgG1 chimeric heterotetramer, the light chains of which are encoded by the expression vector designated CD4-kLC-pRCCMV; or a CD4-IgG1
25 chimeric heterotetramer, both the heavy and the light chains of which are encoded by the above-described expression vectors, in an amount effective to block spread of HIV infection, for example, within the subject or an AIDS patient's body.

30 The invention also provides a pharmaceutical composition which comprises either a CD4-IgG1 chimeric heterotetramer, the heavy chains of which are encoded by the expression vector designated CD4-IgG1HC-pRCCMV; a CD4-IgG1 chimeric
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heterotetramer, the light chains of which are encoded by the expression vector designated CD4-kLC-pRcCMV, or a CD4-IgG1 chimeric heterotetramer, both the heavy and the light chains of which are encoded by the above described expression vectors, in an amount effective to inhibit HIV infection of a CD4+ cell, and a pharmaceutically acceptable carrier.

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Further provided by the invention is a composition of matter comprising either a CD4-IgG1 chimeric heterotetramer, the heavy chains of which are encoded by the expression vector designated CD4-IgG1HC-pRcCMV; a CD4-IgG1 chimeric heterotetramer, the light chains of which are encoded by the expression vector designated CD4-kLC-pRcCMV, or a CD4-IgG1 chimeric heterotetramer, both the heavy and the light chains of which are encoded by the above described expression vectors, and a toxin linked thereto.

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In one embodiment of the invention, the toxin is the deglycosylated A chain of ricin, domains II or III of Pseudomonas exotoxin A, Diphtheria toxin, or a non-peptidyl cytotoxin.

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The invention further provides a diagnostic reagent either comprising a CD4-IgG1 chimeric heterotetramer, the heavy chains of which are encoded by the expression vector designated CD4-IgG1HC-pRcCMV; a CD4-IgG1 chimeric heterotetramer the light chains of which are encoded by the expression vector designated CD4-kLC-pRcCMV; or a CD4-IgG1 chimeric heterotetramer both the heavy and the light chains of which are encoded by both of those expression vectors, and a detectable marker linked thereto. Examples of suitable detectable markers are radioisotopes, chromophores or fluorophores.

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In order to facilitate understanding of the following examples, certain frequently occurring methods and/or terms are best described in Maniatis et al. (37)

5 This invention is illustrated in the Experimental Details section which follows. These sections are set forth to aid in an understanding of the invention but are not intended to, and should not be construed to, limit in any way the invention as set forth in the claims which follow thereafter.

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Experimental Details

A. Materials and Methods

5 1. Construction of CD4-gamma1 chimeric heavy chain gene encoding CD4-gamma1 chimeric heavy chain homodimer:

10 The human CD4 cDNA was excised from the plasmid pSP6T4 (4) as an EcoR1/Stu1 restriction fragment. The 0.70 kilobase fragment was isolated and cloned into EcoR1/Sma1 digested M13mp18. This intermediate vector (M13mp18(CD4)) was then isolated, linearized with Pst1, purified, and treated with Bacterial Alkaline Phosphatase (BAP). The 2.0 Kb Pst1/Pst1 fragment from the plasmid pBr gamma1 containing the human gamma1 heavy chain gene (30), (containing the hinge, CH2, and CH3 exons) was isolated and cloned into the BAP-treated M13mp18/CD4 vector. Resulting recombinants were then screened for the correct orientation of the Pst1 fragment (with respect to the CD4 sequence) to obtain a vector which contains in tandem CD4(EcoR1/Stu1) - gamma1(Pst1/Pst1). To obtain a CD4-gamma1 chimeric heavy chain gene, oligonucleotide-mediated site-directed mutagenesis was performed to juxtapose the CD4 and gamma1 heavy chain DNA sequences, ligating the CD4 sequence in frame to the hinge exon. The resulting chimeric DNA molecule encodes a protein containing the V1V2 domains of CD4 followed by the hinge, CH2, and CH3 domains of gamma1 heavy chain (Figure 1A). Mutagenesis was performed on single-stranded DNA isolated from recombinant phage from transformed TG1 cells (Amersham). Briefly, template DNA was annealed with a 34-mer oligonucleotide (5'-GTCACAAGATTTGGGCTCGAAAGCTAGCACCACG-3'), containing sequences which join the last codon encoding Phe(179) from V1V2 of CD4 to the first codon of the hinge for IgG1 (encoding Glu) (Figures 1A and 3). After second strand synthesis, double stranded DNA was transformed into

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competent TG1 cells. Isolated plaques were then grown in fresh TG1 cells and single stranded DNA was purified for DNA sequencing. All mutations were verified and confirmed by dideoxy sequencing using the Sequenase system (USB). Plaques containing the chimeric gene with the correct sequence were then grown in TG1 cells, and Rf DNA (designated CD4-IgG1-Rf) was isolated from the cells.

2. Construction of Mammalian Expression Vector Encoding CD4-gammal chimeric heavy chain homodimer:

The CD4-gammal chimeric heavy chain gene was isolated from the recombinant Rf DNA following Rf linearization with EcoR1. The EcoR1 sites in the linearized DNA were filled in with the Klenow fragment of DNA polymerase I. The flush ended DNA was then ligated overnight at 15 degrees Celsius with T4 DNA ligase to a 100-fold molar excess of HindIII linkers. After heat inactivation of T4 DNA ligase for 15 minutes at 70 degrees Celsius, the HindIII-linkered DNA was extensively digested with HindIII to liberate a fragment containing the CD4-gammal chimeric heavy chain gene. This HindIII fragment was then purified and ligated to the expression vector pcDNA-1 (Invitrogen), which was previously digested with HindIII and BAP treated. The resulting plasmid was then transformed into MC1061/P3 cells. Plasmid DNA was isolated from recombinant clones, and verification of the presence of the HindIII insert and orientation of the insert with respect to the cytomegalovirus (CMV) promoter in the plasmid was made by restriction enzyme analysis. The resulting mammalian expression plasmid which encodes a CD4-gammal chimeric heavy chain homodimer is designated CD4IgG1-pcDNA1.

3. Expression of CD4-IgG1-pcDNA1 in mammalian cells:

a. Transient expression.

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5 CosM5 cells grown in DMEM containing 10% fetal calf serum were split to 75% confluence. On the following day, the cells were transfected for 16-20 hours with 10 micrograms of CsCl-purified plasmid CD4IgG1-pcDNA1 DNA by the standard CaPO(4) precipitation technique. After transfection, fresh medium was added to the cells. Analysis of the products synthesized 48-72 hours post-transfection was performed by radiolabelling of transfectants with ³⁵S-methionine for 12-18 hours followed by precipitation of media and cell lysates using anti-CD4 antibodies or by incubation with Protein A-sepharose beads alone followed by SDS-PAGE under reducing or non-reducing conditions (Figure 6). In addition, analysis of media and cell lysates was performed 48-72 hours post-transfection by standard Western blotting procedures.

15 b. Stable expression.

Dhfr-Chinese hamster ovary cells (CHO) were transfected with 20 micrograms of CsCl purified DNA in a 1000:1 molar ratio of CD4IgG1-pcDNA1:p410 (p410 is an expression plasmid containing the dhfr gene), although other ratios may also be used. Approximately 3-5 days post-transfection, cells were placed in selective medium (nucleoside-free alpha MEM containing 10% dialyzed fetal calf serum). Approximately 10-15 days post-selection, individual cell clones were picked and analyzed for stable expression of CD4-gammal chimeric heavy chain homodimer by several screening techniques, such as ELISA and precipitation with Protein A-sepharose beads followed by SDS-PAGE under reducing and non-reducing conditions. Clones expressing the highest levels were subjected to successive rounds of amplification of the newly introduced DNA sequences in increasing concentrations of methotrexate. Stable CHO cell lines were thus generated which secrete between 10-100 micrograms/milliliter of CD4-gammal chimeric heavy chain homodimer.

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4. Purification of CD4-gammal chimeric heavy chain homodimer from CHO conditioned media:

CD4-gammal chimeric heavy chain homodimer was purified in a single step using Protein A-Sepharose column chromatography. CHO cells secreting CD4-gammal chimeric heavy chain homodimer were grown to high density in roller bottles in medium containing alpha MEM with 10% IgG-free fetal calf serum. Conditioned media was collected, clarified by centrifugation, and diluted 1:1 with PBS with/or without detergent (i.e. Tween) in this and subsequent buffers. The diluted media was then applied to a 5ml column of Protein A-Sepharose fast flow previously equilibrated with PBS, at a flow rate of 60ml/hour. After extensive washing, the specifically bound material was eluted with 100mM glycine/HCl, pH 3.5, directly into an aliquot of 1M Tris.HCl pH 8.0 to immediately neutralize the eluted fractions. The fractions were then analyzed by SDS-PAGE under reducing and non-reducing conditions followed by silver staining and pooled (Figure 8).

The pooled fractions were then applied to a 10 ml column of S-sepharose fast flow previously equilibrated with 50mM BES pH 7.0 at a flow rate of 120ml/hr. After application of the sample, a step elution gradient (consisting of the following 4 steps: 5 column volumes of 50mM BES pH 7.0, 4 column volumes of 50mM BES pH 7.0, 100mM NaCl, 6 column volumes of 50mM BES pH 7.0 225mM NaCl, followed by 8 column volumes of 50mM BES pH 7.0, 500mM NaCl) was employed for specific elution of the CD4-gammal chimeric heavy chain homodimer. The CD4-gammal chimeric heavy chain homodimer was eluted from the column in 50mM BES pH 7.0, 500mM NaCl. The peak fractions were then pooled and concentrated to yield a final protein concentration of at least 1mg/ml.

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5. Demonstration of binding of CD4-gammal chimeric heavy chain homodimer to the HIV envelope glycoprotein gp120:

5 CosM5 transfectants expressing CD4-gammal chimeric heavy chain homodimer were incubated for 72 hours in DMEM containing 10% IgG-free fetal calf serum. Unlabelled medium was then collected and used to precipitate ³⁵S-methionine-radiolabelled HIV gp120. After incubation of CD4-gammal chimeric heavy chain homodimer containing medium with ³⁵S-methionine-labelled gp120, the complexes were adsorbed to Protein A-sepharose. Protein A-sepharose complexes were recovered by centrifugation, and the precipitates were analyzed by SDS-PAGE under reducing conditions followed by fluorography (Figure 7). Alternatively, aliquots of purified CD4-gammal chimeric heavy chain homodimer from CHO cells were also used to precipitate ³⁵S-radiolabelled gp120 using the same procedure.

6. Determination of plasma half-life and placental transfer of CD4-gammal chimeric heavy chain homodimer:

20 Determination of the plasma half-life and placental transfer are performed by well established techniques. Briefly, rabbits or monkeys are injected intravenously or intramuscularly with purified CD4-gammal chimeric heavy chain homodimer. At various time points post-injection, plasma samples are taken, and the quantity of the CD4-gammal chimeric heavy chain homodimer present in the serum is measured by ELISA. In addition, pregnant monkeys are also injected either IV or IM with CD4-gammal chimeric heavy chain homodimer and the concentration determined in the cord blood and the serum of the newborn monkey. Determination and comparison of the quantity of the CD4-gammal chimeric heavy chain homodimer in the mother's serum as well as in the cord blood and serum of the newborn indicates the

relative rate of transport across the placenta of these molecules.

7. Determination of FcR binding and macrophage infectivity of CD4-gammal chimeric heavy chain homodimer.

5 Determination of FcR binding and macrophage infectivity of CD4-gammal chimeric heavy chain homodimer are performed by well established techniques. For these studies, U937 cells (a human monocytic cell line expressing FcR1 and FcR2),
10 purified monocyte/macrophage populations from human peripheral blood, and Hela cells constitutively expressing recombinant human FcRs are utilized. In addition, monoclonal antibodies specific for FcR1 and FcR2 are commercially available. Briefly, radiolabelled monomeric or aggregated CD4-gammal chimeric heavy chain homodimer is
15 incubated with the above cells and appropriate control cells at 4 degrees Celsius over various time points. At the end of each incubation, the cells are solubized and the cell-associated radioactivity is determined to establish the amount of CD4-gammal chimeric heavy chain homodimer specifically bound to each cell type. As controls,
20 radiolabelled normal monomeric or aggregated human IgG1 are used to determine the levels of specific antibody binding. Furthermore, competing the radiolabelled component with unlabelled monomeric or aggregated normal human IgG1, or
25 monoclonal antibodies to FcR1 or FcR2, will establish the binding efficiency and specificity of CD4-gammal chimeric heavy chain homodimer to each cell type.

30 To ascertain whether the CD4-gammal chimeric heavy chain homodimer mediates enhancement of HIV infection of monocytes/macrophages, HIV-1 is incubated with media alone or either monomeric or aggregated CD4-gammal chimeric heavy chain homodimer at several dilutions. As controls, sera from normal individuals and HIV-infected individuals are
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used (31). After incubation for one hour at 4 degrees Celsius, the 'opsonized' virus is added to the cell types described in the paragraph above. At various time points after infection, the media is harvested and assayed for viral reverse transcriptase activity to determine the degree of viral infection. As controls, sCD4, OKT4a or Leu3a are included during the infection of the cells. In addition, various dilutions of the CD4-gamma1 chimeric heavy chain homodimer and appropriate controls are first incubated with the cells at 4 degrees Celsius to allow binding. HIV is then added and infection assayed by viral reverse transcriptase activity.

8. HIV binding assay:

Binding of HIV was performed as previously described (38, 39). Briefly, concentrated HIV-1 preparations were incubated with various dilutions of sCD4, CD4-gamma1, or CD4-gamma2, for 30 minutes and then added to 5×10^5 CEM cells. Bound virus was detected by indirect immunofluorescence and cytofluorography as previously described (39).

9. Neutralization assay:

The microculture assay for productive viral replication was as previously described (38, 40). Briefly dilutions of sCD4, CD4-gamma1, or CD4-gamma2 were incubated for 30 minutes with 100 TCID₅₀ HIV-1 at room temperature. The mixtures were added to PHA-stimulated lymphocytes and incubated at 37°C overnight. The cells were then washed and plated in microculture at 1×10^5 cells/culture; and 10 cultures per dilution and monitored for reproductive viral replication by detection of HIV antigen in culture supernates 8 and 12 days later.

B. Construction of CD4-IgG1 chimeric heavy chain and CD4-kappa chimeric light chain for expression of CD4-IgG1 chimeric heterotetramer:

1. Introduction

5 This invention describes a CD4-gamma1 chimeric heavy gene encoding a CD4-gamma1 chimeric heavy chain homodimer which is efficiently secreted from transformed mammalian cells. This chimeric molecule was designed to contain sequences from the human IgG1 heavy chain which allow for efficient homodimer assembly and secretion. The CH1 region of the IgG1 heavy chains is responsible for retaining heavy chain molecules intracellularly and for formation of heterotetramers with light chains (25). In order to efficiently produce CD4-gamma1 chimeric heavy chain homodimers, the CD4-gamma1 chimeric heavy chain gene described above specifically lacks the CH1 domain. The resulting homodimer contains two CD4 V1V2 moieties and therefore has the potential of being bivalent with respect to gp120 binding and having enhanced avidity for HIV compared to sCD4.

20 In addition, this invention describes the construction of CD4-IgG1 chimeric heterotetramers which contain two heavy chains and two light chains. The resulting heterotetramer, containing two or four CD4 V1V2 moieties, and has the potential of being tetravalent with respect to gp120 binding and having enhanced avidity for HIV compared to sCD4. The CD4-IgG1 chimeric heavy chain gene used to produce CD4-IgG1 chimeric heterotetramer contains the entire heavy chain constant region, including the CH1 domain. The inclusion of the CH1 domain facilitates efficient intracellular association with light chains, affording the potential for secreted, disulfide-bonded heterotetramers. Both the CD4-IgG1 chimeric heavy chain gene and the CD4-kappa chimeric light chain gene contain the V1V2 domains of CD4. Efforts

to express CD4-IgG1 chimeric heavy chains or CD4-kappa chimeric light chains (either alone or in combination) containing only the V1 domain of CD4 were unsuccessful.

5 2. Construction of CD4-IgG1 chimeric heavy chain expression vector and CD4-kappa chimeric light chain expression vector for production of CD4-IgG1 chimeric heterotetramers.

10 a. Construction of CD4-IgG1 chimeric heavy chain mammalian expression vector.

The human CD4 cDNA sequence is excised from the plasmid pSP6T4 (4) as an EcoR1/StuI restriction fragment. The 0.70 kilobase fragment is isolated and cloned into EcoR1/SmaI-
15 digested M13mp18. The resulting vector (M13mp18(CD4)) is then isolated and digested with BamH1. The BamH1 sites of the M13mp18(CD4) are made flush ended with the Klenow fragment of DNA polymerase 1. After heat inactivation of the polymerase for 15 minutes at 65 degrees Celsius, the
20 linearized M13mp18(CD4) vector is then digested with PstI and purified.

In order to excise a fragment containing the CH1 exon of the human gamma1 heavy chain gene, the plasmid pBr gamma1 (30)
25 is digested with SacII, and the SacII sites are then made flush using T4 DNA polymerase. After heat inactivation of the polymerase, the fragment is then digested with PstI. The resulting SacII(flush)-PstI fragment containing the CH1 exon is then purified and ligated to the M13mp18(CD4) vector
30 described in the above paragraph. After transformation of competent TG1 cells, the resulting recombinants are screened by restriction analysis for the presence of both CD4 and CH1 sequences which contain in tandem CD4 (EcoR1/StuI) - CH1 (SacII(flush)/PstI). Oligonucleotide-mediated site-directed
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mutagenesis is then performed to juxtapose the CD4 and CH1 sequences in frame. The resulting chimeric DNA molecule contains the V1V2 domains of CD4 fused to the CH1 domain of gamma1 heavy chain. Mutagenesis is performed on single-stranded DNA isolated from recombinant phage from transformed TG1 cells (Amersham). Template DNA is annealed with a 33-mer oligonucleotide (5'-GGGCCCTTGGTGGA GGCGAAAGCTAGCACCACG-3') containing sequences which join the last codon encoding Phe (179) from V1V2 of CD4 to the first codon of the CH1 domain for gamma1 heavy chain (encoding Ala). After second strand synthesis, double stranded DNA is transformed into competent TG1 cells. Isolated plaques are then grown in fresh TG1 cells and single-stranded DNA is purified for DNA sequencing. All mutations are confirmed by dideoxy sequencing using the Sequenase system (USB). Plaques containing the chimeric genes with the correct sequence as determined by restriction analysis are then grown in TG1 cells, and the Rf DNA is isolated from the cells.

Rf DNA from the CD4-CH1 chimeric gene is then linearized by digestion with Pst1. The Pst1 linearized vector is then BAP treated and ligated to the Pst1-Pst1 DNA fragment of the plasmid pBr gamma1 containing the hinge, CH2, and CH3 exons of the human gamma1 heavy chain gene. The correct orientation of the Pst1-Pst1 fragment with respect to the chimeric CD4-CH1 fragment is then verified by restriction analysis. The resulting chimeric gene encodes a protein containing the V1V2 domains of CD4 followed by the CH1, hinge, CH2, and CH3 regions of gamma1 heavy chain (Figures 2A, 2B, and 4).

The CD4-IgG1 chimeric heavy chain DNA molecule is isolated from the recombinant Rf DNA following Rf linearization with EcoR1. The EcoR1 sites in the linearized DNA are filled in with the Klenow fragment of DNA polymerase I. The flush

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ended DNA is then ligated overnight at 15 degrees Celsius with T4 DNA ligase to a 100-fold molar excess of HindIII linkers. After heat inactivation of T4 DNA ligase for 15 minutes at 70 degrees Celsius, the HindIII-linkered DNA is extensively digested with HindIII to liberate a fragment containing the CD4-IgG1 chimeric heavy chain gene. This HindIII fragment is then purified and ligated to the expression vector pcDNA-1 (Invitrogen), which was previously digested with HindIII and BAP treated. The resulting plasmid is then transformed into MC1061/P3 cells. Plasmid DNA is isolated from recombinant clones, and verification of the presence of the HindIII insert and orientation of the insert with respect to the cytomegalovirus (CMV) promoter in the plasmid is made by restriction analysis. The resulting mammalian expression plasmid which encodes a CD4-IgG1 chimeric heavy chain is designated CD4-IgG1HC-pRcCMV.

b. Construction of a CD4-kappa chimeric light chain mammalian expression vector:

The human kappa light chain constant region is excised from the plasmid pCNkappa light as an MseI fragment. The purified MseI fragment is then made flush ended using the Klenow fragment of DNA polymerase 1. M13mp18 Rf is then linearized with HincII, and the flush ended MseI kappa light chain fragment is ligated to M13mp18 at the flush ended HincII site in the vector. After transformation of TG1 cells, the recombinants are confirmed for the presence of the insert and the correct orientation within the vector by restriction analysis. Rf is purified from infected TG1 cells and digested with EcoRI and SmaI. The purified vector containing the kappa light chain constant region is then ligated to the EcoRI/StuI fragment of the human CD4 cDNA described above. The resulting recombinants are then verified for the presence and orientation of both inserts

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containing in tandem CD4 (EcoR1/StuI) - Ckappa (MseI(flush)/MseI(flush)), and single-stranded DNA is purified for oligonucleotide-mediated site directed mutagenesis. Template DNA is annealed to a 33-mer oligonucleotide (5'-GATGGTGCAGCCACAGTGAAAGCTAGCACCACG-3') containing sequences which join the last codon encoding Phe(179) from V1V2 of CD4 to the first codon of the kappa light chain constant domain (encoding thr). After second strand synthesis, double-stranded DNA is transformed into competent TG1 cells, and isolated plaques are grown in fresh TG1 cells for DNA sequencing. The presence of the mutation is confirmed by dideoxy sequencing. Plaques containing chimeric genes with the correct sequence are then grown in TG1 cells, and Rf DNA is isolated from the cells. The resulting DNA molecule encodes a protein containing the V1V2 domains of CD4 followed by the constant region of kappa light chains (Figures 2A, 2B and 5).

The CD4-kappa chimeric light chain DNA molecule is isolated from the recombinant Rf DNA following Rf linearization with EcoR1. The EcoR1 sites in the linearized DNA are filled in with the Klenow fragment of DNA polymerase I. The flush ended DNA is then ligated overnight at 15 degrees Celsius with T4 DNA ligase to a 100-fold molar excess of HindIII linkers. After heat inactivation of T4 DNA ligase for 15 minutes at 70 degrees Celsius, the HindIII linkered DNA is extensively digested with HindIII to liberate a fragment containing the CD4-kappa chimeric light chain gene. This HindIII fragment is then purified and ligated to the expression vector pcDNA-1 (Invitrogen), which was previously digested with HindIII and BAP treated. The resulting plasmid is then transformed into MC1061/P3 cells. Plasmid DNA is isolated from recombinant clones, and verification of the presence of the HindIII insert and orientation of the insert with respect to the cytomegalovirus (CMV) promoter in the plasmid is made by restriction enzyme analysis. The

resulting mammalian expression plasmid which encodes a CD4-kappa chimeric light chain is designated CD4-kLC-pRcCMV.

3. Co-expression of CD4-IgG1HC-pRcCMV and CD4-kLC-pRcCMV in mammalian cells to produce CD4-IgG1 chimeric heterotetramer.

a. Transient expression.

CosM5 cells grown in DMEM containing 10% fetal calf serum are split to 75% confluence. On the following day, the cells are transfected for 16-20 hours with 5 micrograms of CsCl purified CD4-IgG1HC-pRcCMV DNA and 5 micrograms of CsCl-purified CD4-kLC-pRcCMV plasmid DNA by the standard CaPO₄(4) precipitation technique. After transfection, fresh medium is added to the cells. Analysis of the products synthesized 48-72 hours post-transfection is performed by radiolabelling of transfectants with ³⁵S-methionine for 12-18 hours followed by precipitation of media and cell lysates using anti-CD4 antibodies or by incubation with Protein A-sepharose beads alone followed by SDS-PAGE under reducing or non-reducing conditions. In addition, analysis of media and cell lysates is performed 48-72 hours post-transfection by standard Western blotting procedures.

b. Stable expression.

Dhfr-Chinese hamster ovary cells (CHO) are transfected with 20 micrograms of CsCl purified DNA in a ratio of 1000:1000:1 CD4-IgG1HC-pRcCMV:CD4-kLC-pRcCMV:p410 (p410 is an expression plasmid containing the dhfr gene), although other ratios may also be used. At approximately 3-5 days post-transfection, cells are placed in selective medium (nucleoside-free alpha MEM containing 10% dialyzed fetal calf serum). At approximately 10-15 days post-selection, individual cell

clones are picked. The clones are then analyzed for stable expression of CD4-IgG1 chimeric heterotetramers by several screening techniques, such as ELISA and precipitation with Protein A-sepharose beads followed by SDS-PAGE under reducing or non-reducing conditions. Clones expressing the highest levels are subjected to successive rounds of amplification of the newly introduced DNA sequences in increasing concentrations of methotrexate. Stable CHO cell lines are thus generated which secrete high levels of CD4-IgG1 chimeric heterotetramer.

4. Purification of CD4-IgG1 chimeric heterotetramers from CHO conditioned media:

CD4-IgG1 chimeric heterotetramers are purified using Protein A-Sepharose column chromatography. CHO cells secreting CD4-IgG1 chimeric heterotetramers are grown to high density in roller bottles in medium containing alpha MEM with 10% IgG-free fetal calf serum. Conditioned media is collected, clarified by centrifugation, and diluted 1:1 with PBS with/or without detergent (i.e. Tween) in this and subsequent buffers. The diluted media is then applied to a 5ml column of Protein A-Sepharose fast flow previously equilibrated with PBS, at a flow rate of 60ml/hour. After extensive washing, the bound material is eluted with 100mM glycine/HCl, pH 3.5, directly into an aliquot of 1M Tris.HCl pH 8.0 to immediately neutralize the eluted fractions. Fractions are then analyzed by SDS-PAGE under reducing and non-reducing conditions followed by silver staining and pooled.

5. Demonstration of binding of CD4-IgG1 chimeric heterotetramer to the envelope glycoprotein gp120:

5 CosM5 transfectants expressing CD4-IgG1 chimeric heterotetramers are incubated for 72 hours in DMEM containing 10% IgG-free fetal calf serum. Unlabelled medium is then collected and used to precipitate ³⁵S-methionine-radiolabelled HIV gp120. After incubation of CD4-IgG1 chimeric heterotetramer containing medium with ³⁵S-methionine-labelled gp120, the complexes are adsorbed to Protein A-sepharose. Protein A-sepharose complexes are recovered by centrifugation, and the precipitates are analyzed by SDS-PAGE followed by fluorography. Alternatively, aliquots of purified CD4-IgG1 chimeric heterotetramers from CHO cells are also used to precipitate ³⁵S-radiolabelled gp120 using the same procedure.

15 6. Determination of plasma half-life and placental transfer of CD4-IgG1 chimeric heterotetramer:

20 Determination of the plasma half-life and placental transfer are performed by well established techniques. Briefly, rabbits or monkeys are injected intravenously or intramuscularly with purified CD4-IgG1 chimeric heterotetramer. At various time points post-injection, plasma samples are taken, and the quantity of the CD4-IgG1 chimeric heterotetramer present in the serum is measured by ELISA. In addition, pregnant monkeys are also injected either IV or IM with CD4-IgG1 chimeric heterotetramer and the concentration determined in the cord blood and the serum of the newborn monkey. Determination and comparison of the quantity of the CD4-IgG1 chimeric heterotetramer in the mother's serum as well as in the cord blood and serum of the newborn indicates the relative rate of transport across the placenta of these molecules.

35 7. Determination of FcR binding and macrophage infectivity of CD4-IgG1 chimeric heterotetramer:

Determination of FcR binding and macrophage infectivity of CD4-IgG1 chimeric heterotetramer are performed by well established techniques. For these studies, U937 cells (a human monocytic cell line expressing FcR1 and FcRII), purified monocyte/macrophage populations from human peripheral blood, and Hela cells constitutively expressing recombinant human FcRs are utilized. In addition, monoclonal antibodies specific for FcR1 and FcRII are commercially available. Briefly, radiolabelled monomeric or aggregated CD4-IgG1 chimeric heterotetramer is incubated with the above cells and appropriate control cells at 4 degrees Celsius over various time points. At the end of each incubation, the cells are solubilized and the cell-associated radioactivity is determined to establish the amount of CD4-IgG1 chimeric heterotetramer specifically bound to each cell type. As controls, radiolabelled normal monomeric or aggregated human IgG1 are used to determine the levels of specific antibody binding. Furthermore, competition of the radiolabelled component with unlabelled monomeric or aggregated normal human IgG1, or monoclonal antibodies to FcR1 or FcRII, will establish the binding efficiency and specificity of CD4-IgG1 chimeric heterotetramer to each cell type.

To ascertain whether the CD4-IgG1 chimeric heterotetramer mediates enhancement of HIV infection of monocytes/macrophages, HIV-1 is incubated with media alone or either monomeric or aggregated CD4-IgG1 chimeric heterotetramer at several dilutions. As controls, sera from normal individuals and HIV-infected individuals are used (31). After incubation for one hour at 4 degrees Celsius, the 'opsonized' virus is added to the cell types described in the paragraph above. At various time points after infection, the media is harvested and assayed for viral reverse transcriptase activity to determine the degree of viral infection. As controls, sCD4, OKT4a or Leu3a are

included during the infection of the cells. In addition, various dilutions of the CD4-IgG1 chimeric heterotetramer and appropriate controls are incubated first with the cells at 4 degrees Celsius to allow binding. HIV is then added and infection assayed by viral reverse transcriptase activity.

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B. Results:

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A CD4-gammal chimeric heavy chain gene encoding a CD4-gammal chimeric heavy chain homodimer was generated by ligating the leader-V1-V2 segment of the human CD4 cDNA (4) to the hinge exon of the human gammal heavy chain gene (30) (Figure 1A). The resulting recombinant DNA molecule (designated CD4-IgG1-Rf) encodes the signal sequence and two amino-terminal immunoglobulin-like domains of the CD4 protein (the first 179 amino acids of mature CD4) followed by the hinge (15 amino acids), CH2 (110 amino acids), and CH3 (107 amino acids) regions of the gammal heavy chain protein (Figure 3). This recombinant DNA molecule also contains two introns present within the gammal heavy chain gene: between the H and CH2 domains, and between the CH2 and CH3 domains. This CD4-gammal chimeric gene was designed to encode a CD4-gammal chimeric heavy chain homodimer which specifically lacks the CH1 domain of the gammal heavy chain. Expression of the CH1 domain without accompanying light chains prevents efficient heavy chain secretion from mammalian cells (25).

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In the CD4-gammal chimeric heavy chain homodimer, the hinge region of one chain contains three cysteine residues, affording the potential of three interchain disulfide bonds (Figure 1B). In contrast, naturally-occurring human IgG1 contains two interchain disulfide bonds between the gammal heavy chains; the amino-terminal cysteine in the gammal hinge region is disulfide bonded to the final cysteine in the light chain constant region, while the two remaining

cysteines in the hinge region form two interchain disulfide bonds between the heavy chains.

The CD4-gammal chimeric heavy chain gene was subcloned into the mammalian expression vector pcDNA1. This vector contains the following DNA elements: the cytomegalovirus (CMV) immediate early promoter and enhancer driving transcription of the CD4-gammal chimeric heavy chain gene; an SV40 polyadenylation sequence; and an SV40 origin of replication which allows replication of the plasmid to high copy number in CosM5 cells. The resulting CD4-gammal heavy chain mammalian expression vector (designated CD4-IgG1-pcDNA1) was transfected into CosM5 cells which were then radiolabelled with ^{35}S -methionine 48-72 hours post-transfection. The radiolabelled medium was analyzed by precipitation with Protein A-sepharose beads and SDS-PAGE followed by fluorography (Figure 6). Under reducing conditions, a protein migrating at a relative molecular mass (Mr) of approximately 47 kilodaltons is precipitated. When the precipitated material was run on SDS-PAGE under nonreducing conditions, a protein migrating at an Mr of approximately 94 kilodaltons is observed, indicating that the CD4-gammal chimeric heavy chains assemble and are secreted as homodimers. In addition, these results demonstrate that the secreted CD4-gammal chimeric heavy chain homodimers contain an intact immunoglobulin Fc domain since they bind Protein A. Further characterization by Western blot analysis of the proteins secreted into the medium 48-72 hours post-transfection was performed using a rabbit polyclonal antiserum raised against purified soluble human CD4. Similar to the results obtained by precipitation, when the medium was run on SDS-PAGE under reducing conditions, followed by Western transfer to nitrocellulose, the major immunoreactive protein migrates at an Mr of approximately 47 kilodaltons. Under nonreducing conditions, the major immunoreactive protein migrates at an

Mr of approximately 94 kilodaltons. Taken together, these results demonstrate that the CD4-gammal chimeric heavy chain is produced and secreted as a homodimer of the predicted molecular weight.

5 The above results demonstrate that the Fc portion of CD4-gammal chimeric heavy chain homodimer, encoded by the constant regions of the gammal heavy chain gene, binds Protein A and is therefore functionally active. In order to determine if the CD4 portion is functionally intact, CD4-gammal chimeric heavy chain homodimers were assayed for
10 their ability to bind to the HIV exterior envelope glycoprotein, gp120 (Figure 7). Unlabelled medium from CosM5 cells transfected with CD4-IgG1-pcDNA1 DNA was incubated with ³⁵S-methionine-labelled gp120. CD4-gammal chimeric heavy chain homodimer/gp120 complexes were
15 precipitated by incubation with Protein A-sepharose beads, and the precipitates were analyzed by SDS-PAGE under reducing conditions followed by fluorography. These results demonstrate that the CD4-gammal chimeric heavy chain homodimer efficiently recognizes HIV gp120 and binds with
20 high affinity. These observations, taken together with the results described in the above paragraph, demonstrate that CD4-gammal chimeric heavy chain homodimer contains functionally active regions of both CD4 and gammal heavy chain.

25 In order to stably produce large quantities of the CD4-gammal chimeric heavy chain homodimers, the CD4-IgG1-pcDNA1 vector was cotransfected with the plasmid p410 (encoding the enzyme dihydrofolate reductase (dhfr)) into dhfr-Chinese
30 Hamster Ovary (CHO) cells. Approximately two weeks post-transfection, individual clones growing in nucleoside free alpha MEM and 10% dialyzed fetal calf serum (and therefore dhfr+) were isolated and analyzed for co-expression of CD4-gammal chimeric heavy chain homodimers by precipitation and
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ELISA. The highest producing cell lines were identified and subjected to stepwise increasing concentrations of methotrexate which selects for amplification of the newly introduced DNA sequences. A CHO cell line expressing 10 micrograms/milliliter of CD4-gammal chimeric heavy chain homodimer was used for stable, constitutive production in roller bottles. The cells were grown to confluence in alpha MEM containing 10% IgG-free fetal calf serum. The cells were then fed every other day and two day old conditioned medium was used for purification of the CD4-gammal chimeric heavy chain homodimer. Conditioned medium was diluted 1:1 with phosphate-buffered saline (PBS) and applied to a 5ml column of Protein A-sepharose fast flow (Pharmacia) at a flow rate of 60 milliliters/hour. The column was then washed with 10 column volumes of PBS and the bound material was eluted with 100 mM glycine pH 3.5. The eluted material was collected directly into 50 μ l of 1M Tris. HCl pH 8.0 to neutralize the eluant. Fractions having an OD(280) of greater than 0.1 were analyzed by SDS-PAGE followed by silver staining or Western blot analysis, and the peak fractions were pooled. A single band was specifically eluted from the Protein A-sepharose column with an Mr corresponding to the CD4-gammal chimeric heavy chain homodimer (Figure 8). Western blot analysis confirms that the eluted protein is immunoreactive with polyclonal antiserum raised against soluble human CD4. In addition, the purified protein retains the ability to bind with high affinity to ³⁵S-methionine-labelled gp120. These results demonstrate the stable, high-level production of CD4-gammal chimeric heavy chain homodimers in mammalian cells, and the purification of CD4-gammal chimeric heavy chain homodimer which retains biological function.

The partially purified CD4-gammal heavy chain homodimer purified as described in Figure 8 was effective at preventing HIV binding to CD4 cells (Figure 9) and

neutralization of infectivity of a fixed HIV inoculum (Figure 10). In this later assay, approximately 10-25 μ g/ml of CD4-gamma1 as well as sCD4 were required to prevent 50% of the cultures from becoming infected by HIV.

5 Further purification of CD4-gamma1 heavy chain homodimer was achieved using ion-exchange chromatography. The peak fraction from the protein A-sepharose column was applied to a 10ml S-sepharose fast flow column preequilibrated with 50mM BES pH 7.0, at a flow rate of 120ml/hr. After application of the sample, the column was extensively washed 10 with 50mM BES pH 7.0 with increasing salt concentration (see materials and methods). A single band of CD4-gamma1 heavy chain homodimer was specifically eluted from the column in 50mM BES pH 7.0 containing 500mM NaCl. These peak fractions were pooled and analyzed by SDS-PAGE and silver staining 15 under non-reducing conditions (Figure 11, lane 1), and reducing conditions (Figure 11, lane 2). When the purified CD4-gamma1 chimeric heavy chain homodimer was run on SDS-PAGE under reducing conditions, a doublet was observed which appeared to be due to differences in glycosylation of the 20 CD4-gamma1 chimeric heavy chain homodimer (data not shown).

A CD4-IgG1HC chimeric heavy chain gene encoding a CD4-IgG1 chimeric heavy chain was generated by ligating the leader-V1-V2 segment of the human CD4 cDNA to the CH1 exon of the 25 human IgG1 heavy chain gene (Figure 2A). In addition, a CD4-kappa chimeric light chain gene encoding a CD4-kappa light chain was generated by ligating the leader-V1-V2 segment of the human CD4 cDNA to the constant domain of the kappa light chain gene (Figure 2A). These CD4-IgG1 chimeric 30 heavy chain genes and CD4-kappa chimeric light chain genes were designed to encode a CD4-IgG1 chimeric heterotetramer, in which the CD4-IgG1 heavy chain contains a CH1 domain for efficient association with kappa light chains.

Both the CD4-IgG1 chimeric heavy chain and the CD4-kappa chimeric light chain genes were subcloned into the mammalian vectors pRcCMV or pPPI-2. Both vectors contain the cytomegalovirus immediate early promoter and enhancer driving transcription of the chimeric genes. In the vector pRcCMV, a second transcriptional cassette which contains the RSV promoter and enhancer is used to direct the transcription of the neomycin resistance gene. In pPPI-2, a second transcriptional cassette which contains the β -globin promoter directs the transcription of the dhfr gene (see supra). In order to stably produce large quantities of the CD4-IgG1 chimeric heterotetramer, the CD4-IgG1 chimeric heavy chain expression vector and the CD4-kappa chimeric light chain expression vector were transfected simultaneously (typically the CD4-IgG1 chimeric heavy chain gene cloned in pRcCMV was used, and CD4-kappa chimeric light chain gene cloned in pPPI-2 was used in a ratio of 1:1). Approximately two weeks post-transfection, individual clones growing in nucleoside-free alpha MEM containing 1 mg/ml G418 and 10% dialyzed fetal calf serum were isolated and analyzed for co-expression of CD4-IgG1 chimeric heavy chains and CD4-kappa chimeric light chains by immunoprecipitation and ELISA. Figure 12 demonstrates one clone selected and analyzed for expression of both CD4-IgG1 chimeric heavy chains and CD4-kappa chimeric light chains. The CHO cell line or the untransfected parental CHO cell line were radiolabelled with ^{35}S -methionine and ^{35}S -cysteine for 16 hours. The radiolabelled medium was analyzed by precipitation with Protein A-sepharose beads and SDS-PAGE under non-reducing conditions followed by fluorography (Figure 12A). Under non-reducing conditions 2 proteins migrating at relative molecular masses of approximately 140 kilodaltons and 210 kilodaltons are precipitated. When the precipitated material was run on SDS-PAGE under non-reducing conditions, 2 proteins migrating at relative molecular masses of 69 kilodaltons and 35 kilodaltons were observed,

which are consistent with the relative predicted molecular masses of the CD4-IgG1 chimeric heavy chains, and CD4-kappa chimeric light chains respectively (data not shown). Further characterization has shown that the protein migrating at 210 kilodaltons on SDS-PAGE under non-reducing conditions contains both CD4-IgG1 chimeric heavy chains and CD4-kappa chimeric light chains which are covalently associated, while the protein migrating at 140 kilodaltons on SDS-PAGE under non-reducing conditions contains only CD4-IgG1 chimeric heavy chains (Figure 12B). These data are consistent with the predicted molecular weight for the 210 kilodalton protein being comprised of 2 CD4-IgG1 chimeric heavy chains and 2 CD4-kappa chimeric light chains, covalently associated to form a molecule with the structure H_2L_2 (H=heavy chain, L=light chain). Furthermore, the 140 kilodalton protein seen on SDS-PAGE under non-reducing conditions is consistent with the predicted molecular weight of a CD4-IgG1 chimeric homodimer with the structure H_2 . Taken together, these results indicate that a CHO cell line which expresses both CD4-IgG1 chimeric heavy chains and CD4-kappa chimeric light chains is able to efficiently assemble and secrete CD4-IgG1 chimeric heterotetramers.

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What is claimed is :

1. An expression vector encoding a CD4-gammal chimeric heavy chain homodimer designated CD4-IgG1-pcDNA1 (ATCC No. 40951).
- 5 2. A CD4-gammal chimeric heavy chain homodimer encoded by the expression vector of claim 1.
3. A method of producing a CD4-gammal chimeric heavy chain homodimer which comprises:
 - 10 a) transfecting a mammalian cell with the expression vector of claim 1;
 - b) culturing the resulting transfected mammalian cell under conditions such that chimeric heavy chain homodimer is produced; and
 - 15 c) recovering the chimeric heavy chain homodimer so produced.
- 20 4. A method of claim 3, wherein the mammalian cell is a COS cell, CHO cell or myeloma cell.
5. A method of inhibiting HIV infection of a CD4+ cell which comprises treating the CD4+ cell with an
25 amount of the CD4-gammal chimeric heavy chain homodimer of claim 2 effective to inhibit infection of the cell.
- 30 6. A method of preventing a subject from being infected with HIV which comprises administering to the subject an amount of the CD4-gammal chimeric heavy chain homodimer of claim 2 effective to prevent the subject from being infected with HIV.

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7. A method of treating a subject infected with HIV so as to block the spread of HIV infection which comprises administering to the subject an amount of the CD4-gammal chimeric heavy chain homodimer of claim 2 effective to block the spread of HIV infection.
- 5
8. A pharmaceutical composition which comprises the CD4-gammal chimeric heavy chain homodimer of claim 2 in an amount effective to inhibit HIV infection of a CD4+ cell and a pharmaceutically acceptable carrier.
- 10
9. A composition of matter comprising a CD4-gammal chimeric heavy chain homodimer of claim 2 and a toxin linked thereto.
- 15
10. A composition of claim 9, wherein the toxin is the deglycosylated A chain of ricin, domains II or III of Pseudomonas exotoxin A, or Diphtheria toxin.
- 20
11. A diagnostic reagent comprising a CD4-gammal chimeric heavy chain homodimer of claim 2 and a detectable marker linked thereto.
- 25
12. A diagnostic reagent of claim 11 wherein the detectable marker is a radioisotope, chromophore, or fluorophore.
- 30
13. An expression vector encoding the heavy chains of a CD4-IgG1 chimeric heterotetramer designated CD4-IgG1HC-pRcCMV (ATCC No. 75192).
- 35
14. An expression vector encoding the light chains of a CD4-IgG1 chimeric heterotetramer designated CD4-kLC-pRcCMV (ATCC No. 75194).

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15. A CD4-IgG1 chimeric heterotetramer, the heavy chains of which are encoded by the expression vector of claim 13.
- 5 16. A CD4-IgG1 chimeric heterotetramer, the light chains of which are encoded by the expression vector of claim 14.
- 10 17. A CD4-IgG1 chimeric heterotetramer the heavy and the light chains of which are encoded by the expression vectors of claims 13 and 14, respectively.
18. A method of producing a CD4-IgG1 chimeric heterotetramer which comprises:
- 15 a) cotransfecting a mammalian cell with the expression vector of claim 13 and an expression vector encoding a light chain;
- 20 b) culturing the resulting cotransfected mammalian cell under conditions such that the CD4-IgG1 chimeric heterotetramer is produced; and
- 25 c) recovering the CD4-IgG1 chimeric heterotetramer so produced.
19. A method of producing an CD4-IgG1 chimeric heterotetramer which comprises:
- 30 a) cotransfecting a mammalian cell with the expression vector of claim 14 and an expression vector encoding an IgG1 heavy chain and;
- 35

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- b) culturing the resulting cotransfected mammalian cell under conditions such that the chimeric heterotetramer is produced; and
- c) recovering the chimeric heterotetramer so produced.
- 5
20. A method of producing a CD4-IgG1 chimeric heterotetramer which comprises:
- 10 a) cotransfecting a mammalian cell with the expression vectors of claim 13 and 14;
- b) culturing the resulting cotransfected mammalian cell under conditions such that the chimeric heterotetramer is produced; and
- 15 c) recovering the chimeric heterotetramer so produced.
- 20 21. A method of claim 18, 19 or 20, wherein the mammalian cell is a COS cell, CHO cell or myeloma cell.
22. A method of inhibiting HIV infection of a CD4+ cell which comprises treating the CD4+ cell with an amount of the CD4-IgG1 chimeric heterotetramer of claim 15, 16 or 17 effective to inhibit infection of the cell.
- 25
23. A method of preventing a subject from being infected with HIV which comprises administering to the subject an amount of the CD4-IgG1 chimeric heterotetramer of claim 15, 16 or 17 effective to prevent the subject from being infected with HIV.
- 30

24. A method of treating a subject infected with HIV so as to block the spread of HIV infection which comprises administering to the subject an amount of CD4-IgG1 chimeric heterotetramer of claim 15, 16 or 17 effective to block spread of HIV infection.

5

25. A pharmaceutical composition which comprises the CD4-IgG1 chimeric heterotetramer of claim 15, 16 or 17 in an amount effective to inhibit HIV infection of a CD4+ cell and a pharmaceutically acceptable carrier.

10

26. A composition of matter comprising a CD4-IgG1 chimeric heterotetramer of claim 15, 16 or 17 and a toxin linked thereto.

15

27. A composition of claim 26, wherein the toxin is the deglycosylated A chain of ricin, domains II or III of Pseudomonas exotoxin A, and Diphtheria toxin.

20

28. A diagnostic reagent comprising a CD4-IgG1 chimeric heterotetramer of claim 15, 16 or 17 and a detectable marker linked thereto.

25

29. A diagnostic reagent of claim 28 wherein the detectable marker is a radioisotope, chromophore or fluorophore.

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Figure 1A

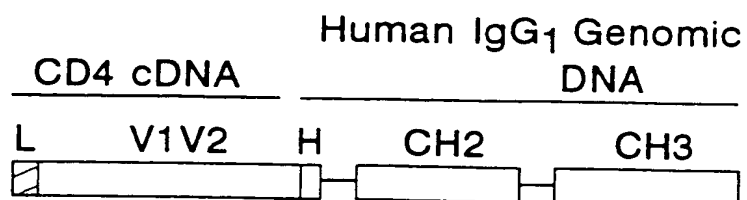
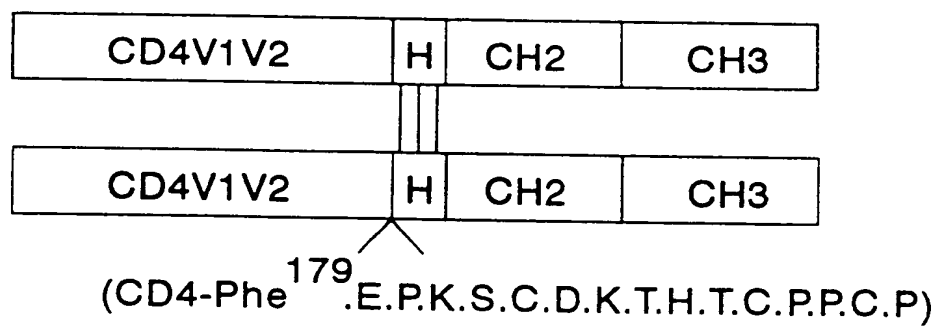


Figure 1B



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Figure 2A

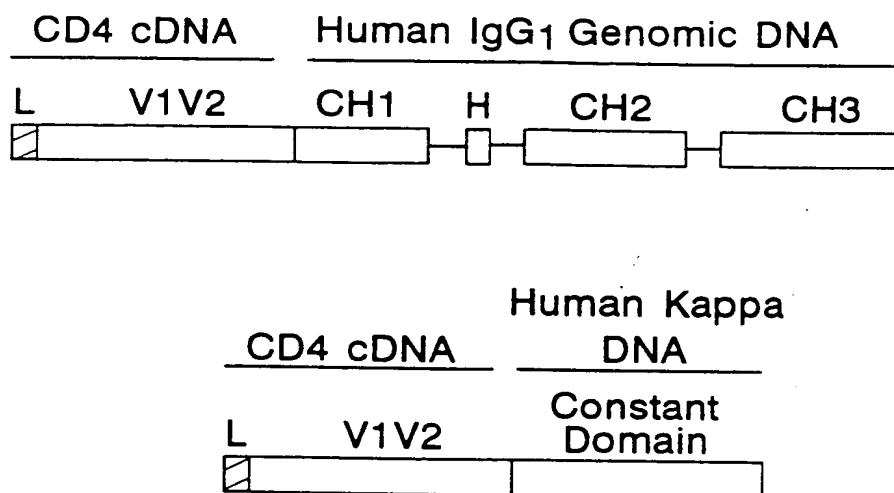
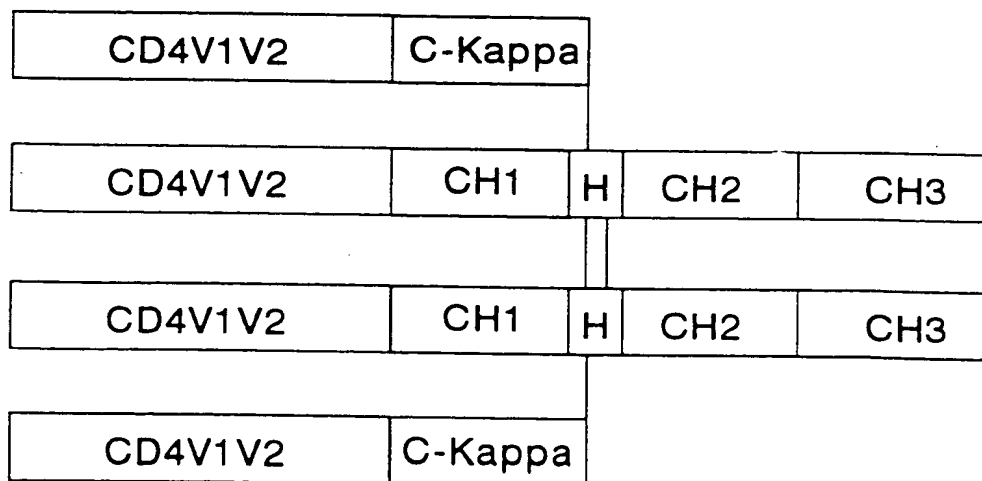


Figure 2B



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Figure 3A

CAAGCCAGAGCCCTGCCATTCTGTGGGCTCAGGTCCCTACTGCTCAGCCCCCTT	55
<div> <div>→CD4</div> <div> <div>M</div> <div>N</div> <div>R</div> <div>G</div> <div>V</div> <div>P</div> <div>F</div> <div>R</div> <div>H</div> </div> </div>	
CCTCCCTCGGCAAGGCCACAATG AAC CGG GGA GTC CCT TTT AGG CAC	102
<div> <div>-10</div> <div> <div>L</div> <div>L</div> <div>L</div> <div>V</div> <div>L</div> <div>Q</div> <div>L</div> <div>A</div> <div>L</div> <div>L</div> <div>P</div> <div>A</div> <div>A</div> <div>T</div> </div> </div>	
TTG CTT CTG GTG CTG CAA CTG GCG CTC CTC CCA GCA GCC ACT	144
<div> <div>-1</div> <div>+1</div> <div> <div>Q</div> <div>G</div> <div>K</div> <div>K</div> <div>V</div> <div>V</div> <div>L</div> <div>G</div> <div>K</div> <div>K</div> <div>G</div> <div>D</div> <div>T</div> <div>V</div> </div> </div>	
CAG GGA AAG AAA GTG GTG CTG GGC AAA AAA GGG GAT ACA GTG	186
<div> <div>+20</div> <div> <div>E</div> <div>L</div> <div>T</div> <div>C</div> <div>T</div> <div>A</div> <div>S</div> <div>Q</div> <div>K</div> <div>K</div> <div>S</div> <div>I</div> <div>Q</div> <div>F</div> </div> </div>	
GAA CTG ACC TGT ACA GCT TCC CAG AAG AAG AGC ATA CAA TTC	228
<div> <div>+30</div> <div> <div>H</div> <div>W</div> <div>K</div> <div>N</div> <div>S</div> <div>N</div> <div>Q</div> <div>I</div> <div>K</div> <div>I</div> <div>L</div> <div>G</div> <div>N</div> <div>Q</div> </div> </div>	
CAC TGG AAA AAC TCC AAC CAG ATA AAG ATT CTG GGA AAT CAG	270
<div> <div>+50</div> <div> <div>G</div> <div>S</div> <div>F</div> <div>L</div> <div>T</div> <div>K</div> <div>G</div> <div>P</div> <div>S</div> <div>K</div> <div>L</div> <div>N</div> <div>D</div> <div>R</div> </div> </div>	
GGC TCC TTC TTA ACT AAA GGT CCA TCC AAG CTG AAT GAT CGC	312

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Figure 3B

																+60																
A	D	S	R	R	S	L	W	D	Q	G	N	F	P																			
GCT	GAC	TCA	AGA	AGA	AGC	CTT	TGG	GAC	CAA	GGA	AAC	TTC	CCC																			
														+70																		
L	I	I	K	N	L	K	I	E	D	S	D	T	Y																			
CTG	ATC	ATC	AAG	AAT	CTT	AAG	ATA	GAA	GAC	TCA	GAT	ACT	TAC																			
														+80																		
I	C	E	V	E	D	Q	K	E	E	V	Q	L	L																			
ATC	TGT	GAA	GTG	GAG	GAC	CAG	AAG	GAG	GAG	GTG	CAA	TTG	CTA																			
														+90																		
														+100																		
V	F	G	L	T	A	N	S	D	T	H	L	L	Q																			
GTG	TTC	GGA	TTG	ACT	GCC	AAC	TCT	GAC	ACC	CAC	CTG	CTT	CAG																			
														+110																		
														+120																		
G	Q	S	L	T	L	T	L	E	S	P	P	G	S																			
GGG	CAG	AGC	CTG	ACC	CTG	ACC	TTG	GAG	AGC	CCC	CCT	GGT	AGT																			
														+130																		
S	P	S	V	Q	C	R	S	P	R	G	K	N	I																			
AGC	CCC	TCA	GTG	CAA	TGT	AGG	AGT	CCA	AGG	GGT	AAA	AAC	ATA																			

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Figure 3C

+140 Q G G G G G G A A G A A C C T C T C C G T G T C T S V S Q L E L Q
 C A G G G G G A A G A A C C T C T C C G T G T C T C A G C T G G A G C T C C A G 606

+150

D S G G T W T C T V L Q N Q K
 G A T A G T G G C A C C T G G A C A T G C A C T G T C T T G C A G A A C C A G A A G 648

+160

K V E F K I D I V V L A F E
 A A G G T G G A G T T C A A A A T A G A C A T C G T G G T G C T A G C T T T C G A G 690

+170

P K S C D K T H T C P P C P
 C C C A A A T C T T G T G A C A A A A C T C A C A C A T G C C C A C C G T G C C C A 732

+180

G G T A A G C C A G C C C C T C G C C C T C C A G C T C A A G C G G G A C A G G T G C C C T A G A G 787

+190

T A G C C T G C A T C C A G G A C A G G C C C C A G C C G G G T G T G A C A C G T C C A C C T C C A T C T 842

+200

C T T C C T C A G C A C C T G A A C T C C T G G G G G A C C G T C A G T C T C T T C C T C 886

→CH2

→Hinge

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Figure 3D

F	P	P	K	P	K	D	T	L	M	I	S	R	T	+220	
TTC	CCC	CCA	AAA	CCC	AAG	GAC	ACC	CTC	ATG	ATC	TCC	CGG	ACC		928
P	E	V	T	C	V	V	V	D	V	S	H	E	D		
CCT	GAG	GTC	ACA	TGC	GTG	GTG	GTG	GAC	GTG	AGC	CAC	GAA	GAC		970
P	E	V	K	F	N	W	Y	V	D	G	V	E	V		
CCT	GAG	GTC	AAG	TTC	AAC	TGG	TAC	GTG	GAC	GGC	GTG	GAG	GTG		1012
H	N	A	K	T	K	P	R	E	E	Q	Y	N	S		
CAT	AAT	GCC	AAG	ACA	AAG	CCG	CGG	GAG	GAG	CAG	TAC	AAC	AGC		1054
T	Y	R	V	V	S	V	L	T	V	L	H	Q	D		
ACG	TAC	CGG	GTG	GTC	AGC	GTC	CTC	ACC	GTC	CTG	CAC	CAG	GAC		1096
W	L	N	G	K	E	Y	K	C	K	V	S	N	K		
TGG	CTG	AAT	GGC	AAG	GAG	TAC	AAG	TGC	AAG	GTC	TCC	AAC	AAA		1138

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Figure 3E

A	L	P	A	P	I	E	K	T	I	S	K	A	K	1180
+300														
GCC	CTC	CCA	GCC	CCC	ATC	GAG	AAA	ACC	ATC	TCC	AAA	GCC	AAA	
GGTGGG	ACCCGTGGGG	TGCGAGGG	CCACATGGACAGAG	CCGGCTCGGCC	ACCC	1235	<div>└→CH3</div>							
TCTGCC	CTGAGAGT	GACCGCTGTACCA	ACCTCTGTCTCTACAGGG	CAG	CCC	CGA	1288	<div>G Q P R</div>						
+310														
E	P	Q	V	Y	T	L	P	P	S	R	D	E	L	
GAA	CCA	CAG	GTG	TAC	ACC	CTG	CCC	CCA	TCC	CGG	GAT	GAG	CTG	1330
+320														
T	K	N	Q	V	S	L	T	C	L	V	K	G	F	
ACC	AAG	AAC	CAG	GTC	AGC	CTG	ACC	TGC	CTG	GTC	AAA	GGC	TTC	1372
+330														
Y	P	S	D	I	A	V	E	W	E	S	N	G	Q	
TAT	CCC	AGC	GAC	ATC	GCC	GTG	GAG	TGG	GAG	AGC	AAT	GGG	CAG	1414
+340														
+350														
+360														
P	E	N	N	Y	K	T	T	P	P	V	L	D	S	
CCG	GAG	AAC	AAC	TAC	AAG	ACC	ACG	CCT	CCC	GTG	CTG	GAC	TCC	1456
+350														
+360														

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Figure 3F

D G S F F L Y S K L T V D K
 GAC GGC TCC TTC TTC CTC TAC AGC AAG CTC ACC GTG GAC AAG 1498
 +370
 S R W Q Q G G N V F S C S V M
 AGC AGG TGG CAG CAG GGG AAC GTC TTC TCA TGC TCC GTG ATG 1540
 +380
 H E A L H N H Y T Q K S L S
 CAT GAG GCT CTG CAC AAC CAC TAC ACG CAG AAG AGC CTC TCC 1582
 +400
 L S P G K stop
 CTG TCT CCG GGT AAA TGAGTGCACGGCCGGCAAGCCCCGCTCCCCGGGC 1632
 +410
 TCTCGCGGTGCACGAGGATGCTTGGCACGTACCCCCCTGTACATACTTCCCGGC 1687
 GCCCAGCATGGAAATAAAGCACCCAGCGCTGCCCTGGGCCCCCTGCGAGACTGTGA 1742
 TGGTTCTTCCACGGGTCAGGCCGAGTCTGAGGCCCTGAGTGGCATGAGGGAGGCA 1797
 GAGCGGGTC... 1806

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Figure 4A

CAAGCCAGAGCCCTGCCATTCTGTGGCTCAGTCCCTACTGCTCAGCCCCCTT	55
<div> <div> <div>→CD4</div> <div> <div>M</div> <div>N</div> <div>R</div> <div>G</div> <div>V</div> <div>P</div> <div>F</div> <div>R</div> <div>H</div> </div> </div> <div>-20</div> </div>	102
CCTCCCTCGGCAAGGCCACAATG AAC CGG GGA GTC CCT TTT AGG CAC	
<div> <div>-10</div> <div> <div>L</div> <div>L</div> <div>V</div> <div>L</div> <div>Q</div> <div>L</div> <div>A</div> <div>L</div> <div>L</div> <div>P</div> <div>A</div> <div>A</div> <div>T</div> </div> </div>	144
TTG CTT CTG GTG CTG CAA CTG GCG CTC CTC CCA GCA GCC ACT	
<div> <div>-1</div> <div>+1</div> <div> <div>Q</div> <div>G</div> <div>K</div> <div>K</div> <div>V</div> <div>V</div> <div>L</div> <div>G</div> <div>K</div> <div>K</div> <div>G</div> <div>D</div> <div>T</div> <div>V</div> </div> </div>	186
CAG GGA AAG AAA GTG GTG CTG GGC AAA AAA GGG GAT ACA GTG	
<div> <div>+20</div> <div> <div>E</div> <div>L</div> <div>T</div> <div>C</div> <div>T</div> <div>A</div> <div>S</div> <div>Q</div> <div>K</div> <div>K</div> <div>S</div> <div>I</div> <div>Q</div> <div>F</div> </div> </div>	228
GAA CTG ACC TGT ACA GCT TCC CAG AAG AAG AGC ATA CAA TTC	
<div> <div>+30</div> <div> <div>H</div> <div>W</div> <div>K</div> <div>N</div> <div>S</div> <div>N</div> <div>Q</div> <div>I</div> <div>K</div> <div>I</div> <div>L</div> <div>G</div> <div>N</div> <div>Q</div> </div> </div>	270
CAC TGG AAA AAC TCC AAC CAG ATA AAG ATT CTG GGA AAT CAG	
<div> <div>+50</div> <div> <div>G</div> <div>S</div> <div>F</div> <div>L</div> <div>T</div> <div>K</div> <div>G</div> <div>P</div> <div>S</div> <div>K</div> <div>L</div> <div>N</div> <div>D</div> <div>R</div> </div> </div>	312
GGC TCC TTC TTA ACT AAA GGT CCA TCC AAG CTG AAT GAT CGC	

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Figure 4B

+60
 A D S R R S L W D Q G N F P
 GCT GAC TCA AGA AGC CTT TGG GAC CAA GGA AAC TTC CCC 354
 +70
 L I I K N L K I E D S D T Y
 CTG ATC ATC AAG AAT CTT AAG ATA GAA GAC TCA GAT ACT TAC 396
 +80
 +90
 I C E V E D Q K E E V Q L L
 ATC TGT GAA GTG GAG GAC CAG AAG GAG GAG GTG CAA TTG CTA 438
 +100
 V F G L T A N S D T H L L Q
 GTG TTC GGA TTG ACT GCC AAC TCT GAC ACC CAC CTG CTT CAG 480
 +110
 +120
 G Q S L T L T L E S P P G S
 GGG CAG AGC CTG ACC CTG ACC TTG GAG AGC CCC CCT GGT AGT 522
 +130
 S P S V Q C R S P R G K N I
 AGC CCC TCA GTG CAA TGT AGG AGT CCA AGG GGT AAA AAC ATA 564

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Figure 4C

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+140      +150
Q  G  G  K  T  L  S  V  S  Q  L  E  L  Q
CAG GGG GGG AAG ACC CTC TCC GTG TCT CAG CTG GAG CTC CAG      606

      +160
D  S  G  G  T  W  T  C  T  V  L  Q  N  Q  K
GAT AGT GGC ACC TGG ACA TGC ACT GTC TTG CAG AAC CAG AAG      648
      CH1
      +180
K  V  E  F  K  I  D  I  V  V  L  A  F  A
AAG GTG GAG TTC AAA ATA GAC ATC GTG GTG CTA GCT TTC GCC      690

      +190
S  T  K  G  P  S  V  F  P  L  A  P  S  S
TCC ACC AAG GGC CCA TCG GTC TTC CCC CTG GCA CCC TCC TCC      732

      +200
K  S  T  S  G  G  T  A  A  L  G  C  L  V
AAG AGC ACC TCT GGG GGC ACA GCG GCC CTG GGC TGC CTG GTC      774

+210      +220
K  D  Y  F  P  E  P  V  T  V  S  W  N  S
AAG GAC TAC TTC CCC GAA CCG GTG ACG GTG TCG TGG AAC TCA      816

      +230
G  A  L  T  S  G  V  H  T  F  P  A  V  L
GGC GCC CTG ACC AGC GGC GTG CAC ACC TTC CCG GCT GTC CTA      858

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Figure 4D

+240
 Q S S G L Y S L S S V V T V +250
 CAG TCC TCA GGA CTC TAC TCC CTC AGC AGC GTG GTG ACC GTG 900

 P S S S L G T Q T Y I C N V
 CCC TCC AGC AGC TTG GGC ACC CAG ACC TAC ATC TGC AAC GTG 942

 +260
 N H K P S N T K V D K K V
 AAT CAC AAG CCC AGC AAC ACC AAG GTG GAC AAG AAA GTTGGTGA 986

 +270
 GAGGCCAGCACAGGGAGGGGTGTCTGTGGAAGCAGGCTCAGCGCTCCTGCC 1041

 TGGACGCATCCCGGCTATGCAGCCCCAGTCCAGGGCAGCAAGCAGGCCCGTCT 1096

 GCCTCTTCACCCGGAGCCTCTGCCCCGCCCCACTCATGCTCAGGGAGAGGTTCTC 1151

 TGGCTTTTCCAGGCTCTGGGCAGGCACAGGCTAGGTGCCCCCTAACCCAGGCC 1206

 TGCACACAAAGGGCAGGTGCTGGGCTCAGACCTGCCAAGAGCCATATCCGGGAG 1261

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Figure 4E

GACCCGTGCCCCCTGACCTAAGCCCAACCCCAAGGCCAACTCTCCACTCCCTCAGC 1316
 TCGGACACCTTCTCTCCTCCAGATTCCAGTAAGTCCCAATCTTCTCTCTGCAGAG 1372
 E
 →Hinge
 +280
 P K S C D K T H T C P P C P
 CCC AAA TCT TGT GAC AAA ACT CAC ACA TGC CCA CCG TGC CCA 1414
 GGTAAGCCAGCCAGGCTCGCCCTCCAGCTCAAGCGGGACAGGTGCCCTAGAG 1469
 TAGCCTGCATCCAGGACAGGCCCCAGCCGGGTGCTGACACGTCACCTCCATCT 1524
 →CH2
 A P E L L G G G P S V F L
 CTTCCCTCAGCA CCT GAA CTC CTG GGG GGA CCG TCA GTC TTC CTC 1568
 +310
 F P P K P K D T L M I S R T
 TTC CCC CCA AAA CCC AAG GAC ACC CTC ATG ATC TCC CGG ACC 1610
 +320
 P E V T C V V D V S H E D
 CCT GAG GTC ACA TGC GTG GTG GTG GAC GTG AGC CAC GAA GAC 1652
 +330

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Figure 4F

```

P   E   V   K   F   N   W   Y   V   D   G   V   E   V
CCT GAG GTC AAG TTC AAC TGG TAC GTG GAC GGC GTG GAG GTG 1694

+340

H   N   A   K   T   K   P   R   E   E   Q   Y   N   S
CAT AAT GCC AAG ACA AAG CCG CGG GAG GAG CAG TAC AAC AGC 1736

+350

T   Y   R   V   V   S   V   L   T   V   L   H   Q   D
ACG TAC CGG GTG GTC AGC GTC CTC ACC GTC CTG CAC CAG GAC 1778

+370

W   L   N   G   K   E   Y   K   C   K   V   S   N   K
TGG CTG AAT GGC AAG GAG TAC AAG TGC AAG GTC TCC AAC AAA 1820

+380

A   L   P   A   P   I   E   K   T   I   S   K   A   K
GCC CTC CCA GCC CCC ATC GAG AAA ACC ATC TCC AAA GCC AAA 1862

+390

GGTGGACCCGTGGGTGCCGAGGGCCACATGGACAGAGCCGGCTCGGCCACCC 1917
      |
      |→CH3
      G   Q   P   R
TCTGCCCTGAGAGTGACCGCTGTACCAACCTCTGTCTCTACAGGG CAG CCC CGA 1970

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Figure 4G

E	P	Q	V	Y	T	L	P	P	S	R	D	E	L	+420	
GAA	CCA	CAG	GTG	TAC	ACC	CTG	CCC	CCA	TCC	CGG	GAT	GAG	CTG		2012
T	K	N	Q	V	S	L	T	C	L	V	K	G	F	+430	
ACC	AAG	AAC	CAG	GTC	AGC	CTG	ACC	TGC	CTG	GTC	AAA	GGC	TTC		2054
Y	P	S	D	I	A	V	E	W	E	S	N	G	Q	+440	
TAT	CCC	AGC	GAC	ATC	GCC	GTG	GAG	TGG	GAG	AGC	AAT	GGG	CAG		2096
P	E	N	N	Y	K	T	T	P	P	V	L	D	S	+450	
CCG	GAG	AAC	AAC	TAC	AAG	ACC	ACG	CCT	CCC	GTG	CTG	GAC	TCC	+460	2138
D	G	S	F	F	L	Y	S	K	L	T	V	D	K	+470	
GAC	GGC	TCC	TTC	TTC	CTC	TAC	AGC	AAG	CTC	ACC	GTG	GAC	AAG		2180
S	R	W	Q	Q	G	N	V	F	S	C	S	V	M	+480	
AGC	AGG	TGG	CAG	CAG	GGG	AAC	GTC	TTC	TCA	TGC	TCC	GTG	ATG	+490	2222
H	E	A	L	H	N	H	Y	T	Q	K	S	L	S	+500	
CAT	GAG	GCT	CTG	CAC	AAC	CAC	TAC	ACG	CAG	AAG	AGC	CTC	TCC		2264

Figure 4H

L S P G K stop
CTG TCT CCG GGT AAA TGAGTGGACGGCCGGCAAGCCCCGCTCCCCGGGC 2313

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TCTCGCGGTCCGACGAGGATGCTTGGCACGTACCCCCCTGTACATACTTCCCCGGGC 2368

GCCCCAGCATGGAAATAAAGCACCCAGCGCTGCCCTGGGCCCCCTGCCGAGACTGTGA 2423

TGGTTCTTCCACGGGTCAGGCCGAGTCTGAGGCCCTGAGTGGCATGAGGGAGGCA 2478

GAGCGGGTC...

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Figure 5A

[illegible]

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Figure 5B

A	D	S	R	R	S	L	W	D	Q	G	N	F	P	
GCT	GAC	TCA	AGA	AGA	AGC	CTT	TGG	GAC	CAA	GGA	AAC	TTC	CCC	354
L	I	I	K	N	L	K	I	E	D	S	D	T	Y	
CTG	ATC	ATC	AAG	AAT	CTT	AAG	ATA	GAA	GAC	TCA	GAT	ACT	TAC	396
I	C	E	V	E	D	Q	K	E	E	V	Q	L	L	
ATC	TGT	GAA	GTG	GAG	GAC	CAG	AAG	GAG	GAG	GTG	CAA	TTG	CTA	438
V	F	G	L	T	A	N	S	D	T	H	L	L	Q	
GTG	TTC	GGA	TTG	ACT	GCC	AAC	TCT	GAC	ACC	CAC	CTG	CTT	CAG	480
G	Q	S	L	T	L	T	L	E	S	P	P	G	S	
GGG	CAG	AGC	CTG	ACC	CTG	ACC	TTG	GAG	AGC	CCC	CCT	GGT	AGT	522
S	P	S	V	Q	C	R	S	P	R	G	K	N	I	
AGC	CCC	TCA	GTG	CAA	TGT	AGG	AGT	CCA	AGG	GGT	AAA	AAC	ATA	564

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Figure 5C

```

+140      +150
Q   G   G   K   T   L   S   V   S   Q   L   E   L   Q
CAG GGG GGG AAG ACC CTC TCC GTG TCT CAG CTG GAG CTC CAG      606

      +160
D   S   G   T   W   T   C   T   V   L   Q   N   Q   K
GAT AGT GGC ACC TGG ACA TGC ACT GTC TTG CAG AAC CAG AAG      648
      +170      +180      +190
K   V   E   F   K   I   D   I   V   V   L   A   F   T
AAG GTG GAG TTC AAA ATA GAC ATC GTG GTG CTA GCT TTC ACT      690
      +200      +210      +220
V   A   A   P   S   V   F   I   F   P   P   S   D   E
GTG GCT GCA CCA TCT GTC TTC ATC TTC CCG CCA TCT GAT GAG      732

Q   L   K   S   G   T   A   S   V   V   C   L   L   N
CAG TTG AAA TCT GGA ACT GCC TCT GTT GTG TGC CTG CTG AAT      774

      +210      +220
N   F   Y   P   R   E   A   K   V   Q   W   K   V   D
AAC TTC TAT CCC AGA GAG GCC AAA GTA CAG TGG AAG GTG GAT      716

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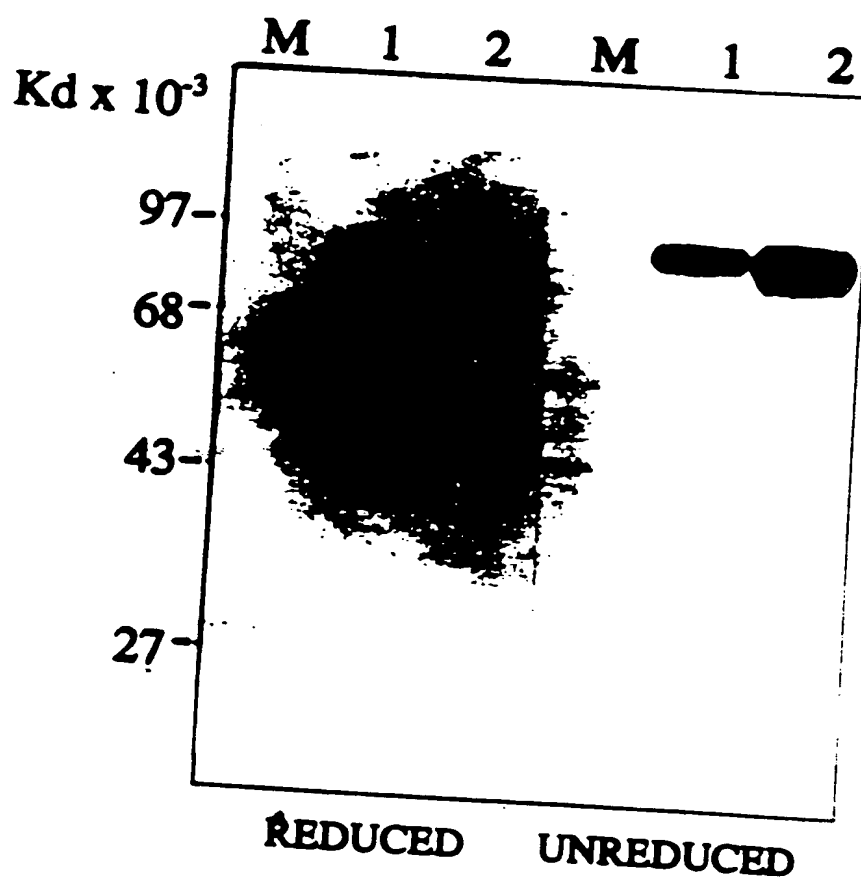
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Figure 5D

N A L Q S G N S Q E S V T E
 AAC GCC CTC CAA TCG GGT AAC TCC CAG GAG AGT GTC ACA GAG 758
 +230
 Q D S K D S T Y S L S S T L
 CAG GAC AGC AAG GAC AGC ACC TAC AGC CTC AGC AGC ACC CTG +250
 900
 T L S K A D Y E K H K V Y A
 ACG CTG AGC AAA GCA GAC TAC GAG AAA CAC AAA GTC TAC GCC +260
 942
 C E V T H Q G L S S P V T K
 TGC GAA GTC ACC CAT CAG GGC CTG AGC TCG CCC GTC ACA AAG 984
 +270
 +280
 S F N R G E C stop
 AGC TTC AAC AGG GGA GAG TGT TAG AGGGAGAAGTGCCCCACCTGCTC 1032
 CTCAGTTCAGCCTGACCCCCCTCCCATCCTTTGGCCTCTGACCCCTTTTCCACAGG 1088
 GGACCTACCCCTATTGCGGGTCCCTCCAAGCTCATCTTTACACCTCACCCCCCTCCTCC 1144
 TCCTT

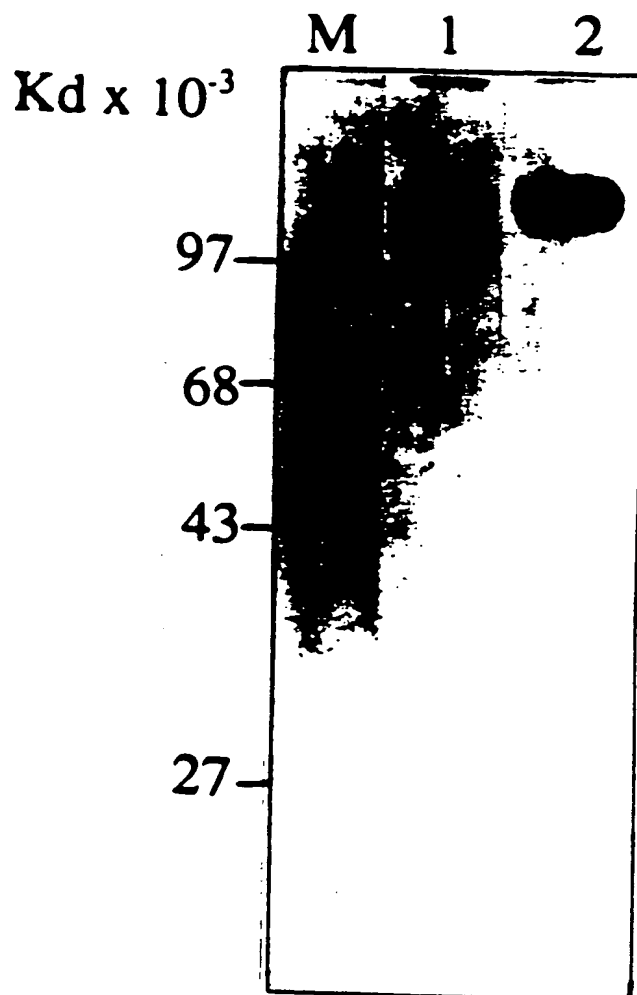
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Figure 6



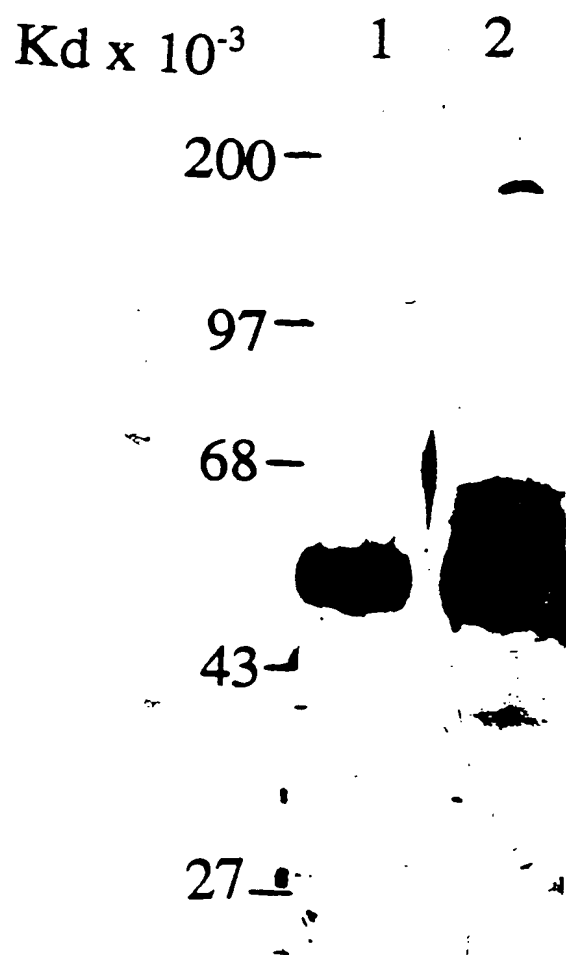
22/27

Figure 7



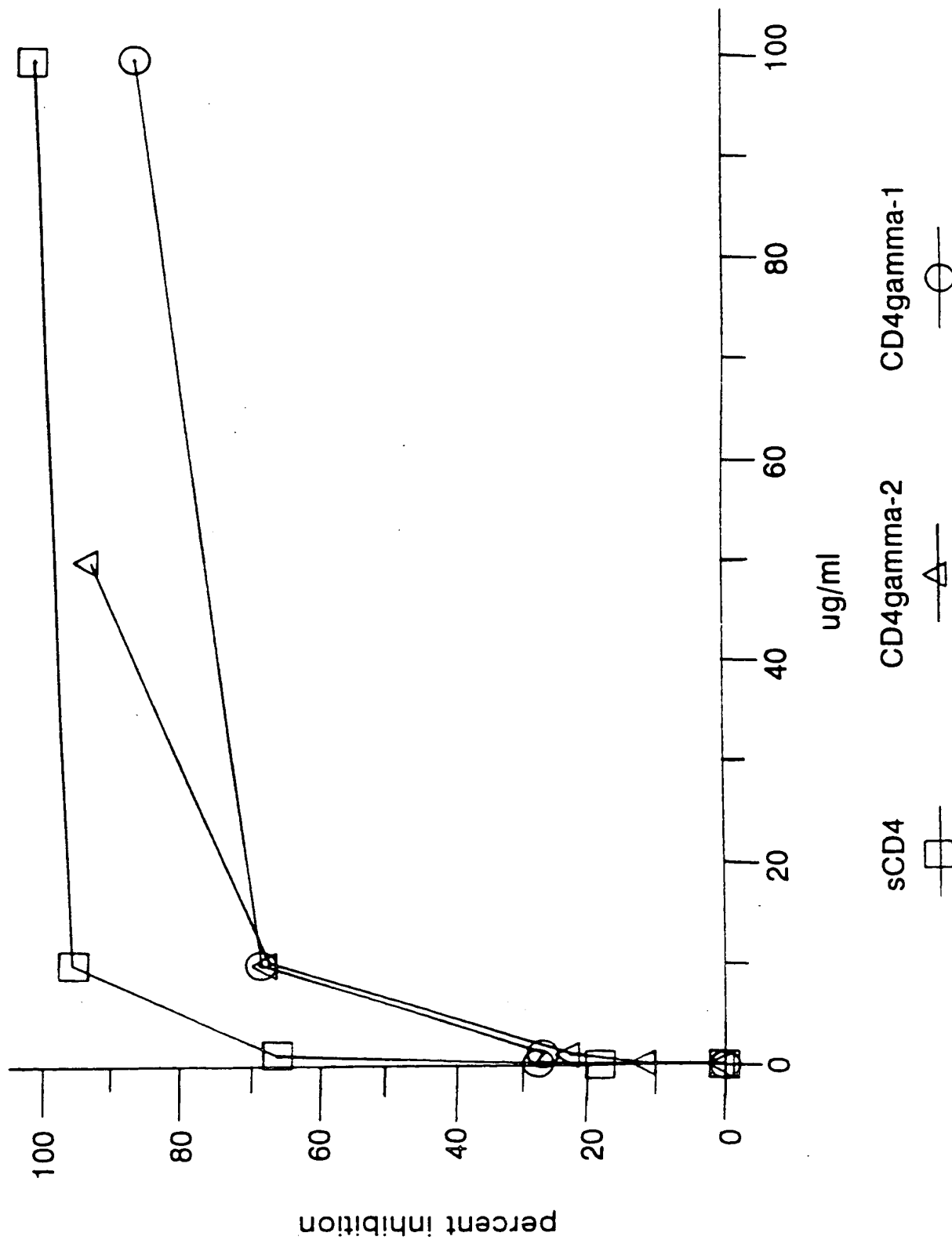
23/27

Figure 8



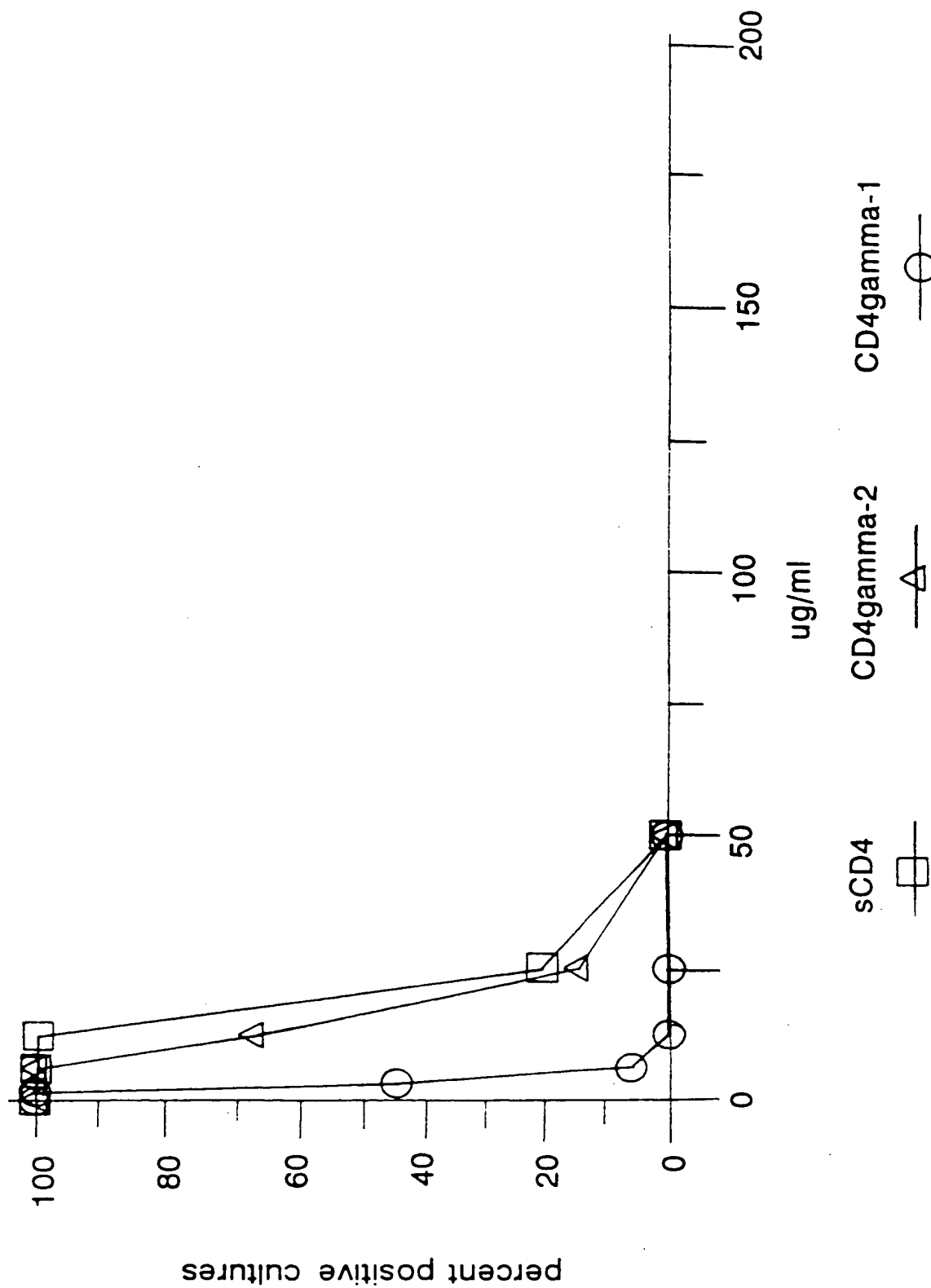
SUBSTITUTE SHEET

Figure 9



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Figure 10



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Figure 11

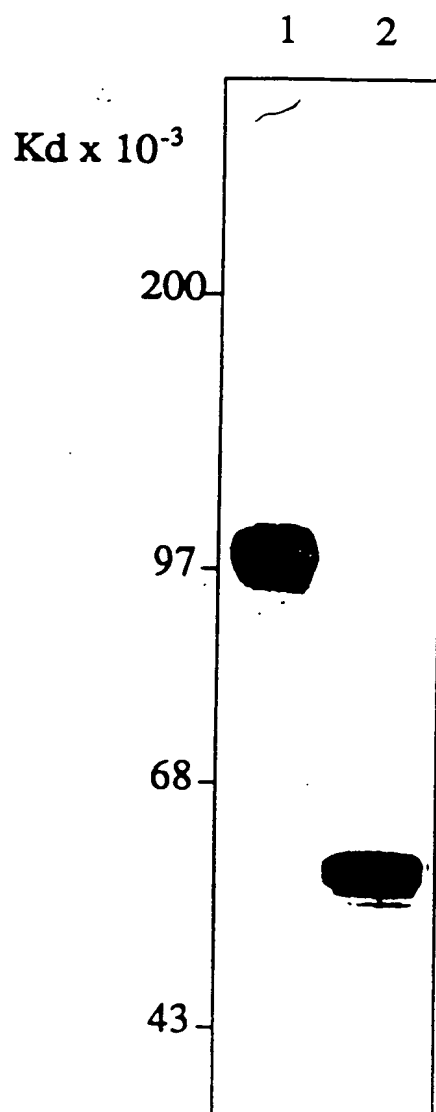


Figure 12B

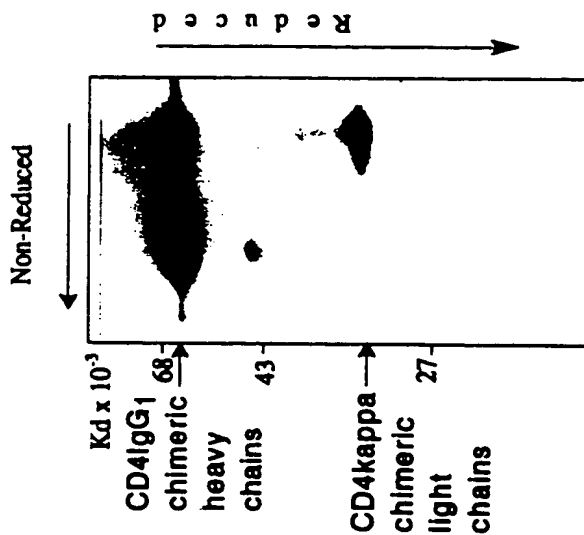
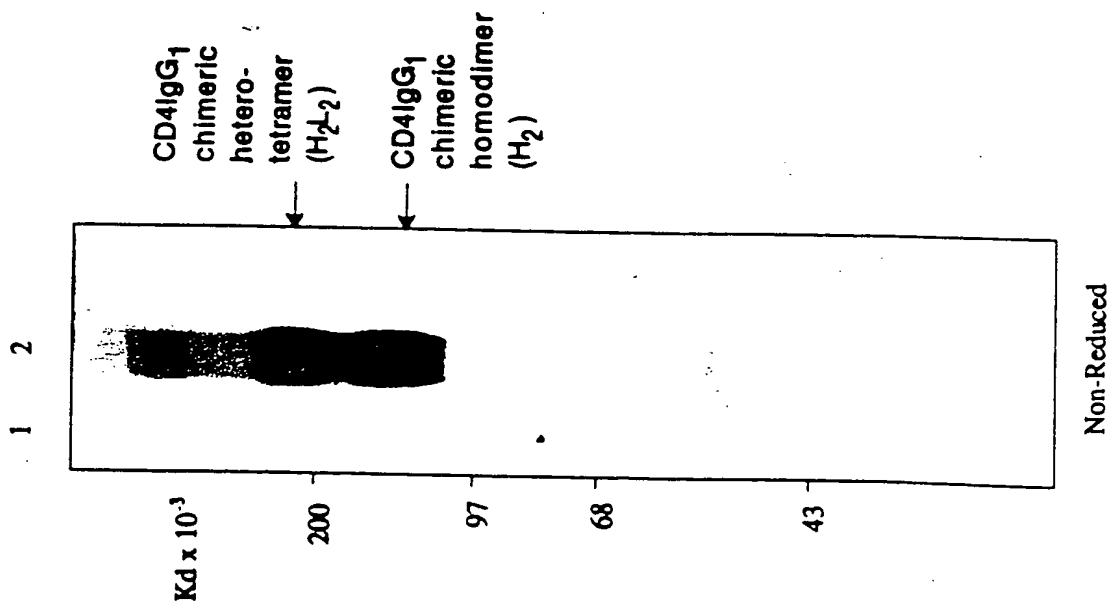


Figure 12A



INTERNATIONAL SEARCH REPORT

International Application No. PCT/US92/01152

I. CLASSIFICATION OF SUBJECT MATTER (if several classification symbols apply, indicate all) ³		
According to International Patent Classification (IPC) or to both National Classification and IPC IPC (5): Please See Attached Sheet. US CL : Please See Attached Sheet.		
II. FIELDS SEARCHED		
Minimum Documentation Searched ⁴		
Classification System	Classification Symbols	
U.S.	530/387.3, 391.1, 191.3, 391.7, 866; 435/ 69.3, 69.7; 424/85.8, 85.9, 85.91; 436/ 514; 935/12, 15	
Documentation Searched other than Minimum Documentation to the extent that such Documents are included in the Fields Searched ⁵		
Please See Attached Sheet.		
III. DOCUMENTS CONSIDERED TO BE RELEVANT ¹⁴		
Category*	Citation of Document, ¹⁶ with indication, where appropriate, of the relevant passages ¹⁷	Relevant to Claim No. ¹⁸
X Y	NATURE, VOLUME 339, ISSUED 04 MAY 1989, TRAUNCKER ET AL, "HIGHLY EFFICIENT NEUTRALIZATION OF HIV WITH RECOMBINANT CD4-IMMUNOGLOBULIN MOLECULES", PAGES 68-70, SEE ENTIRE DOCUMENT.	1-4,13-21 8-12,25-29
X Y	WO, A, 89/03222 (REINHERZ ET AL) 20 APRIL 1989, SEE ENTIRE DOCUMENT.	1-4,13-21 8-12,25-29
Y	WO, A, 88/01304 (MADDON ET AL) 25 FEBRUARY 1988, SEE ENTIRE DOCUMENT.	1-4,8-21, 25-29
X Y	EP, A, 0,314,317 (CAPON ET AL) 03 MAY 1989,SEE ENTIRE DOCUMENT.	1-4,13-21 8-12,25-29
Y	CELL, VOLUME 42, ISSUED AUGUST 1985, MADDON ET AL, "THE ISOLATION AND NUCLEOTIDE SEQUENCE OF A cDNA ENCODING THE T CELL SURFACE PROTEIN T4: A NEW MEMBER OF THE IMMUNOGLOBULIN GENE FAMILY", PAGES 93-104, SEE ENTIRE DOCUMENT.	1-4,8-21, 25-29
X Y	WO, A, 89/02922, (CAPON ET AL) 06 APRIL 1989, SEE ENTIRE DOCUMENT.	1-4,13-21 8-12,25-29
X Y	WO, A, 89/01940, (FISHER ET AL) 09 MARCH 1989, SEE ENTIRE DOCUMENT.	1-4,13-21 8-12,25-29
<div style="display: flex; justify-content: space-between;"> <div style="width: 45%;"> <p>* Special categories of cited documents:¹⁶</p> <p>"A" document defining the general state of the art which is not considered to be of particular relevance</p> <p>"E" earlier document but published on or after the international filing date</p> <p>"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)</p> <p>"O" document referring to an oral disclosure, use, exhibition or other means</p> <p>"P" document published prior to the international filing date but later than the priority date claimed</p> </div> <div style="width: 45%;"> <p>"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention</p> <p>"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step</p> <p>"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art</p> <p>"&" document member of the same patent family</p> </div> </div>		
IV. CERTIFICATION		
Date of the Actual Completion of the International Search ²		Date of Mailing of this International Search Report ²
07 MAY 1992		11 JUN 1992
International Searching Authority ¹		Signature of Authorized Officer ^{2b}
ISA/US		T. MICHAEL NISBET

FURTHER INFORMATION CONTINUED FROM THE SECOND SHEET

V. ☐ OBSERVATIONS WHERE CERTAIN CLAIMS WERE FOUND UNSEARCHABLE¹

This international search report has not been established in respect of certain claims under Article 17(2) (a) for the following reasons:

1. ☐ Claim numbers __, because they relate to subject matter (1) not required to be searched by this Authority, namely:

2. ☐ Claim numbers __, because they relate to parts of the international application that do not comply with the prescribed requirements to such an extent that no meaningful international search can be carried out (1), specifically:

3. ☐ Claim numbers __, because they are dependent claims not drafted in accordance with the second and third sentences of PCT Rule 6.4(a).

VI. ☒ OBSERVATIONS WHERE UNITY OF INVENTION IS LACKING²

This International Searching Authority found multiple inventions in this international application as follows:

Please See Attached Sheet.

1. ☐ As all required additional search fees were timely paid by the applicant, this international search report covers all searchable claims of the international application.

2. ☒ As only some of the required additional search fees were timely paid by the applicant, this international search report covers only those claims of the international application for which fees were paid, specifically claims:

(telephone practice) 1-4, 8-21, 25-29

3. ☐ No required additional search fees were timely paid by the applicant. Consequently, this international search report is restricted to the invention first mentioned in the claims; it is covered by claim numbers:

4. ☐ As all searchable claims could be searched without effort justifying an additional fee, the International Search Authority did not invite payment of any additional fee.

Remark on protest

☐ The additional search fees were accompanied by applicant's protest.

☐ No protest accompanied the payment of additional search fees.

FURTHER INFORMATION CONTINUED FROM PREVIOUS SHEETS

I. CLASSIFICATION OF SUBJECT MATTER:

IPC (5):

A61K 39/00, 35/14; C12P 21/06; G01N 33/558; C07K 15/00, 13/00; C07H 15/12

I. CLASSIFICATION OF SUBJECT MATTER:

US CL :

530/387.3, 391.1, 391.3, 391.7, 866; 435/ 69.3, 69.7; 424/85.8, 85.9, 85.91; 436/514; 935/12, 15; 536/27

II. FIELDS SEARCHED

Other Documents Searched:

AUTOMATED PATENT SYSTEM, FILE: USPAT; DIALOG ONLINE ONESEARCH; FILE BIOSIS, MEDLINE, BIOTECHNOLOGY ABSTRACTS, EMBASE, WORLD PATENT INDEX
 KEYWORDS: CD4, HIV, FUSION OR HETEROLOGOUS() PROTEIN OR PEPTIDE OR POLYPEPTIDE, IMMUNOTOXIN, RICN, DIPHTHERIA, TOXIN?

VI. OBSERVATIONS WHERE UNITY OF INVENTION WAS LACKING

This ISA found multiple inventions as follows:

I. CLAIMS 1,3,4,13-14, AND 18-21 DRAWN TO EXPRESSION VECTORS AND METHODS OF USING THOSE VECTORS CATEGORIZED AS A FIRST APPEARING PRODUCT AND A FIRST APPEARING METHOD OF USING THAT PRODUCT.

II. CLAIMS 2,8-12, 15-17, AND 25-29 DRAWN POLYPEPTIDES, PHARMACEUTICALS, IMMUNOTOXINS, AND DIAGNOSTICS FOR THE PROTEINS ENCODED BY THE VECTORS OF GROUP I. THE PROTEINS AND DERIVATIVES THEREOF ARE SECOND APPEARING PRODUCTS.

III. CLAIMS 5 AND 22 ARE A SECOND APPEARING METHOD OF USING THE PROTEINS OF GROUP II FOR INHIBITING HIV INFECTION.

IV. CLAIMS 6 AND 23 ARE A THIRD APPEARING METHOD OF USING THE PROTEINS OF GROUP II FOR PREVENTING HIV INFECTION.

V. CLAIMS 7 AND 24 ARE A FOURTH APPEARING METHOD OF USING THE PROTEINS OF GROUP II FOR TREATING SUBJECTS INFECTED WITH HIV.